10/500988

IL 03/00026

PA 996918

THE UNITED STATES OF AMERICA

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UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

April 18, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/391,575*
FILING DATE: *June 27, 2002*

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
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By Authority of the
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ASSIGNMENT

For good and valuable consideration, the receipt and sufficiency of which is hereby acknowledged, the undersigned:

Amir Arav Meir Uri

(hereinafter called the "assignor(s)"), hereby sell(s), assign(s) and transfer(s) to:

Interface Multigrad Technology Ltd.
3 Hamazmera St.
Ness Ziona 70400
Israel

(hereinafter called the "assignee(s)"), its/his successors, assignees, nominees or other legal representatives, the Assignor's entire right, title and interest in and to the invention entitled:

EMBRYO GUARD

described and claimed in the following patent applications:

U.S. Provisional Application identified as Attorney docket No. 791/14 and executed the same date as this assignment;

and in and to said Patent Applications, and all original and reissued Patents granted therefor, and all divisions and continuations thereof, including the right to apply and obtain Patents in all other countries, the priority rights under International Conventions, and the Letters Patent which may be granted thereon;

Signed and sealed this 27 day of June 2002

Amir Arav

Meir Uri

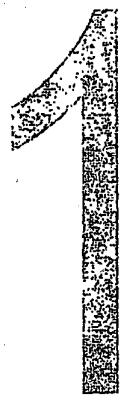
UNITED STATES PATENT AND TRADEMARK OFFICE
DOCUMENT CLASSIFICATION BARCODE SHEET

As-Filed New Application

Level -1
Version 1.1
Updated - 8/01/01

UNITED STATES PATENT AND TRADEMARK OFFICE
DOCUMENT CLASSIFICATION BARCODE SHEET

Transmittal



Level - 2
Version 1.1
Updated - 8/01/01

27/06 '02 14:12 [redacted]

60391575.062702 A/PROV

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Docket Number: 791/14

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
AMIR MEIR	ARAV URI.	BET DAGAN, ISRAEL BET HASHITA, ISRAEL

☐ Additional inventors are being named on page 2 attached hereto

TITLE OF THE INVENTION (280 characters max)

EMBRYO GUARD

CORRESPONDENCE ADDRESS

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☐ Customer Number [ ] — Place Customer Number Bar Code Label here

OR

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification — Number of Pages [ ]

☐ Drawing(s) — Number of Sheets [ ]

Applicant is Small Entity

☒ Other (specify) Assignment

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

☐ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 06-2140

FILING FEE AMOUNT: $80 +40

The Invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: [ ]

Respectfully submitted,

SIGNATURE [ ] DATE 27 JUN 02

TYPED or PRINTED NAME Mark M. Friedman REGISTRATION NO. (if appropriate) 33,883

TELEPHONE (703) 415-1561

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231

(Page 1 of 1)

PTO/SB/16 (8-00)

06/27/02 111126 U.S. PTO

06/27/02 JC973 U.S. PTO 60/391575

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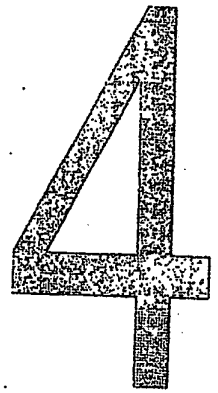
UNITED STATES PATENT AND TRADEMARK OFFICE
DOCUMENT CLASSIFICATION BARCODE SHEET

Specification



Level 2
Version 1.1
Updated - 8/01/01

June 2002

IMT Ltd.

Microscopic Monitoring

Technology and Products

Microscopic Monitoring

During its many years of research in the fields of cryopreservation and reproduction, IMT has developed several techniques and supporting technologies to improve its research process. The MMS technology is a result of one of these developments.
Initially, the company's researchers needed a tool that allows microscopic monitoring of cells during the freezing process. The solution was a miniature device, consisting of a CCD camera, a microscopic long-distance objective lens and a special adapter. That was the beginning of a new field for IMT and based on that simple device, the company develops several important products, including the **EmbryoGuard**.

1. The Technology

Microscopic monitoring is very common in biology, as it is a basic tool for most procedures. In every lab there are usually several types of microscopes and most of the devices have a special adapter for video or CCD camera.
However, this simple approach has several limitations. First, the image must go through the optical system of the microscope. This means that the microscope should be a high quality device, so that the quality of imaging remains untouched. Such high quality microscopes are very costly. In addition, the size of the microscope limits its applications. If one wants to monitor cell development inside an incubator, he can "build" an incubator that surrounds an exiting microscope, or he can take the cells out of the incubator and place them under a microscope in a warm environment.
IMT has succeeded in miniturizing the whole system, while avoiding the need for a microscope. A very small CCD or video camera, with a special adapter and a microscopic lens, can provide the same results as an expensive and high quality system.
An improvement to this basic technology was introduced when the Company developed a robotic system that can control multi-sample monitoring, with X-Y-Z micro movement. This system is based on one or two microscopic CCD cameras, together with robotic features that can move the cameras and the samples. This system is then placed inside a standard incubator. A control unit placed outside the incubator helps to control focusing and illumination, changing between samples, and more. The

control unit can be handled manually or by computer software and the images are screened on a standard monitor.

2. Products and Applications

The Company is developing several products based on that technology. The main one is the Embryo Guard.

The Embryo Guard (EG) is a robotic system for microscopic monitoring and control over embryo development during IVF procedure. It has computerized control and software that assists with embryo evaluation, supports the selection process, and controls the matching process (patient/oocyte/sperm/embryo).

During IVF procedure, it is extremely important to monitor the development of fertilized eggs, from the moment of fertilization up to the stage when 2-3 embryos are selected for transplantation. The importance of this monitoring is derived from the fact that eventually, the best embryos should be chosen for transplantation. Therefore, the clinician must watch very carefully every development stage of the embryos.

Problems being addressed

Change in conditions - Currently, the monitoring procedure is done manually, by taking the embryos out of the incubator, placing them under a microscope and investigating their development.

This approach has several disadvantages that usually damage the embryos. To assure the best conditions for embryo development, it is essential that the embryo remains in a stable controlled environment, as provided by the incubator. Any change in these conditions can easily harm the embryo. Therefore, the procedure of taking the embryo out of the incubator, although it is necessary, has a bad effect on the embryo development. In addition, the optimal way to evaluate embryo development is to monitor it every 3 hours, but again, since this monitoring might be too risky, most IVF labs prefer to perform this evaluation much less frequently. Another problem raised from the need to monitor an embryo under a microscope is that the embryo must be under a special solution (oil) that can damage it.

The EG solves these problems by providing continuous monitoring of the embryo, without taking it out of the incubator. The EG, automatically, monitors each embryo

every 3 hours, or continuously (time-laps recording) and stores this data (as image files) on the embryo records. This process is done inside the incubator which means that embryos do not experience any change in condition and also, there is no need to use oil or any other solution that may cause damage.

Another important advantage of the EG is the ability to control it from a distance, using the Internet. The embryo specialist does not have to be present in the IVF lab, and he can control the whole process using a standard computer connected to the Internet.

Standards and data recording - Another problem addressed is the lack of standards and data recording. Currently, there is no software application that supports the IVF procedure in terms of embryo development and selection of eggs and embryo. Without such supporting software, IVF labs collect data on the embryos in a variety of ways, with no specific standard or quality control, and in addition, most of this data collection is done by paper work.

The EG includes a software that satisfies these needs. Each embryo in the incubator has its own record, containing all the information from the initial stage. The software also automatically collects and stores pictures of each embryo in each stage. In addition to data collecting, the software also helps to evaluate the embryo by indicating in which stage it should be, how many cells it should have, what should be the next stage and the timing for this stage, etc. It can also provide a multi-embryo screen that helps to compare their visual shape.

Matching – IVF labs pay a lot of attention to the issue of matching between oocytes and sperm, or embryos and patient. Even a minor mistake could be a personal disaster for the future parents and a major legal problem to the lab. One component of the EG aims to solve this problem. The EG has a unique matching system that makes sure that no mistake can happen. Every sperm sample and every oocyte are labeled (on the dish) with a barcode labeling system. This procedure is then stored in the computer record as the first step of the IVF procedure. From the moment of labeling, every procedure must first go through a barcode reader that stores the information under the patient record. Before fertilization, the EG software identifies both patients and indicates if there is matching or not. The fertilized eggs are then placed inside the incubator (again - after going through the barcode reader). If during the incubation

period, or even before transplanting the embryos, the clinician needs to take the embryos outside the incubator, the software identifies the specific dish and let the clinician take only this one.

Schedule

IMT plan to introduce the first commercial version of the EG on July 2002.

Consumable products

The Embryo Guard can handle up to 12(?) dishes simultaneously. Each dish is for one specific patient and it can contain up to 10(?) embryos. The dish is a regular and standard dish, sterilized, and is currently available in the market.

For locating and identifying the embryos in specific locations around the dish, the company offers a special sticker for each dish, which also contains barcode ID. The EG cannot operate without IMT's stickers.

Embryo Guard

Notes from interview with Amir Arav, 26 June 27-06-2002

Based on US 6,166,761. Now full robotic system.

Point 1: Follow development of embryos because in vitro fertilization depends on first cleavage. Need to know timing of cleavage. Implant three-day-old embryos. Hard to tell which one to pick.

Point 2:
Prior art: embryos removed from incubator to exchange medium.
New: automatic medium exchange. Add medium, remove medium, or do both. Gas the medium before or after warming up the medium.

Point 3: Zona pellucida stays thick in an incubator. As a result, the embryo may die. Focus a laser beam through a microscope to heat the zona pellucida to cut it open. Do this inside the incubator. Cutting may be done manually by technician, or automatically. Prior art is to remove embryo from incubator and cut zona pellucida outside incubator.

Point 4: Fluorescent markers. Also for preimplementation diagnostics.

Point 5: Insemination inside incubator.

Point 6: Preparation for cryopreservation: is a special case of Point 1.

In general: micromanipulation of oocytes and embryos is done inside the incubator.

Matching using bar code (or equivalent: remotely readable chip, imaging, etc.)
Identify gametes, oocytes, sperm upon collection.
Stickers on test tubes, vials, petri dishes (containers generally). ID text (parents names etc.) matched automatically to ID code.

Box outside incubator has place for one test tube and one petri dish.
Match test tube to petri dish based on bar code. Match embryos and cryogenic vials (need liquid-nitrogen-resistant bar code) for cryopreservation. Match again when transferring embryos to womb.
Gamete Intra Fallopian Transfer. Zygote Intra Fallopian Transfer. Match at Pre-Implantation Diagnosis.

Management software: tells you whatto do when (timing is critical). Warns if embryos are outside incubator too long. Collect history automatically.

To be able to handle 12 petri dishes in the same incubator:
Automatic orientation
CCD camera goes from drop to drop automatically
Search for embryo automatically or go to center of drop
Embryos can move, so image at least 10 times the area of an embryo
Digital magnification. Use high resolution CCD.

The Embryo Guard also can be used for other applications which require culturing cells or tissues in an incubator for a long period of time and to monitor the cells or tissue without removing them from the incubator.

Patent on EmbryoGuard

1.On line monitoring, time laps reordering, medium change over and assisted hatching of embryos inside the incubator,

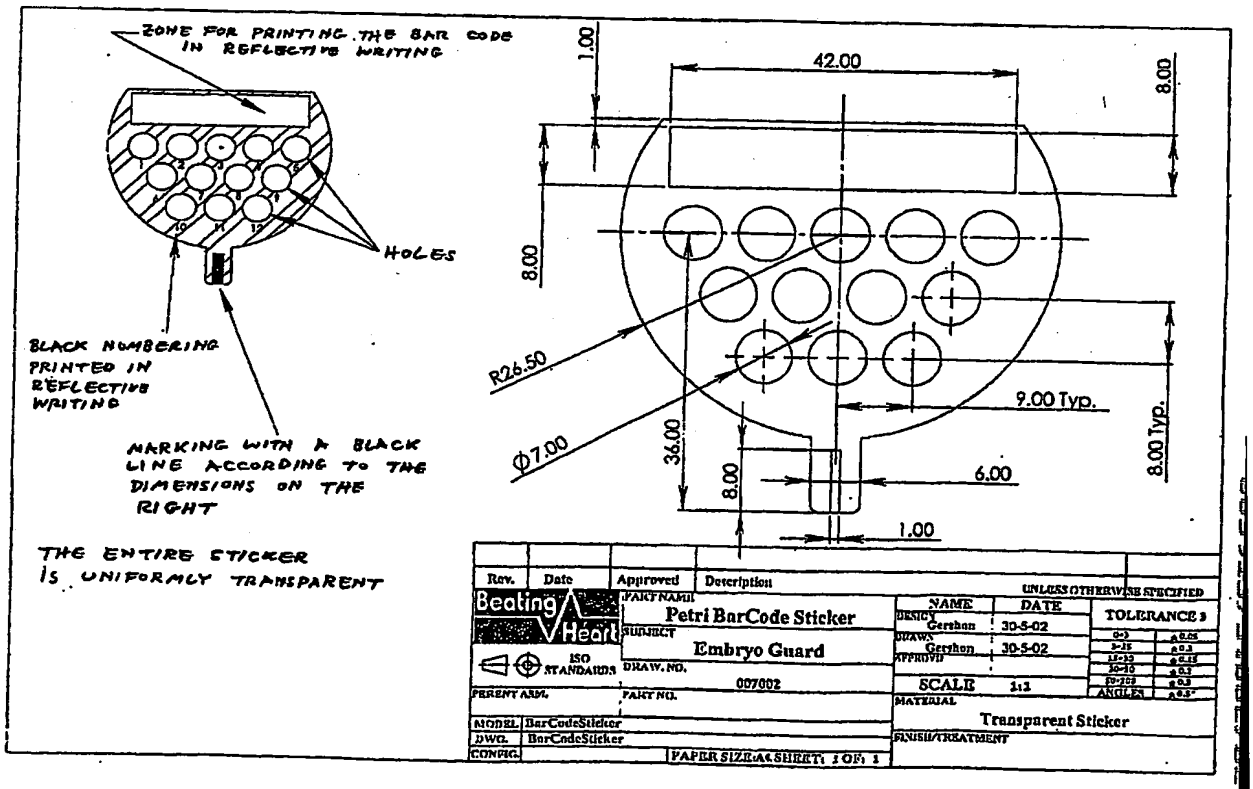
It is been well recognized that the timing of the first cleavage and the morphology of the embryos determine the successful of the IVF procedure in addition, change over of medium and assisted hatching are other reasons for removal the embryos from the incubator which could be optimized if they could be performed inside the incubatore. Many opening of the incubator i.e. for microscopic evaluation, medium change-over and assisted hatching, will affect the embryos culture condition (temperature, gas concentration and humidity).
We describe here a robotic system which will operate in the incubator with the following feature:
1. A microscopic follow up of the development of embryos inside the incubator with the following possibility: A. A real-time evaluation of the embryos using up to 4 different microscope CCDs which could be operate on 3D movements also by using internet compatibility. B. automatic photographing system for image of up to 12 different dishes in which 12 drops are placed in each of the Petri dish.
2. Medium change over is done by computerized injection to each of the drops with small volume of 1-10 microliter of fresh medium which is maintained cooled before injection and then warmed up, gassed and added to the drop.
3. Assisted hatching is done by laser beam performing on the zona pelucida in order to assist blastocyst hatching.
4. Evaluation using flouroscent markers which are loaded with the injectors and are detected by the embryoguard.
5. Insemination could be done stepwise by the injectors insert sperms directly to the drops.
6. preparation oocytes or embryos for cryopreservation inside the drops using the computerized injector in a stepwise manner and according to the osmotic behavior of the cells.

2. Control matching using barcode system.

It is estimate that there are hundreds of mistakes in IVF matching worldwide.
Identification of embryos is done by the technicians created humanity mistakes.
We propose of using a adhesive sticker with a barcode for test tube and Petri dish.
The EmbryoGaurd read the barcode and identify the oocyte for matching with sperm
In a separate apparatus which is placed outside of the incubator (an EmbryoSeater).
The matching could be performed in several levels:

1. inside the incubator before the oocytes are fertilitized.
2. when sperm arrive to the lab
3. between sperm and oocytes
4. for PGD
5. for cryopreservation

In a case of no matching the EmbryoGuard will not aloud to be open and remove out the oocytes or embryos or done any other function.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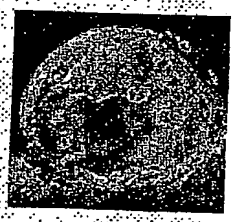
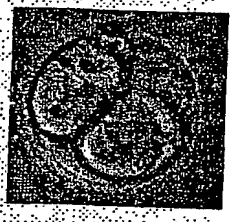
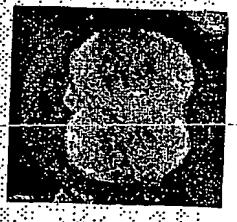
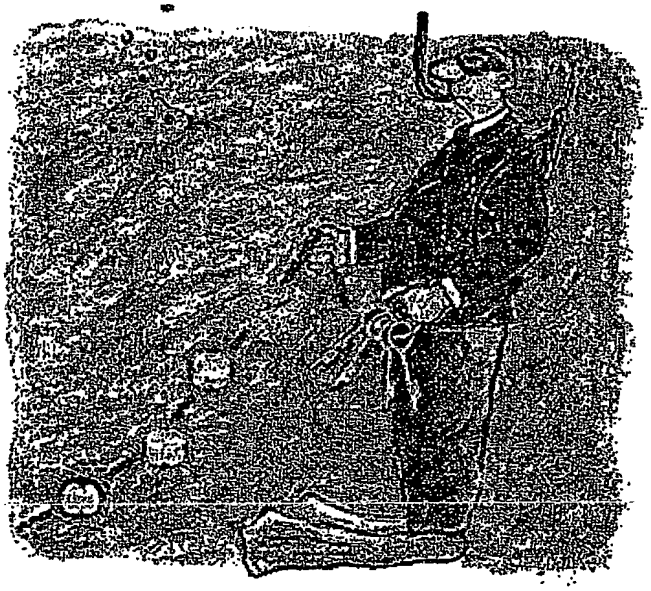
I.M.T LTD
Embryo Guard

The EMBRYOGUARD



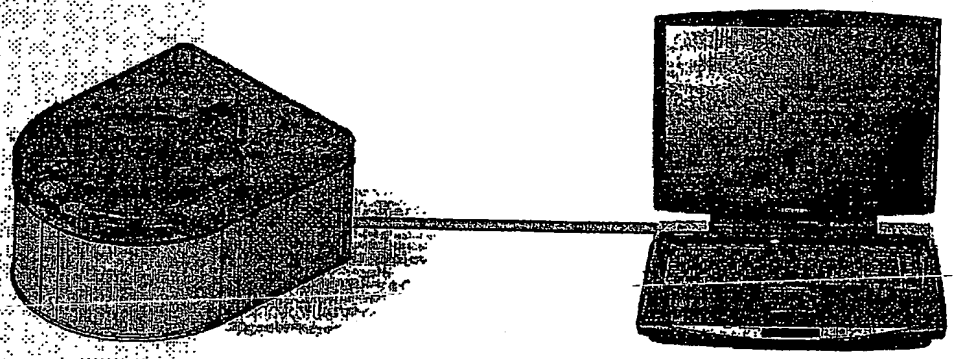




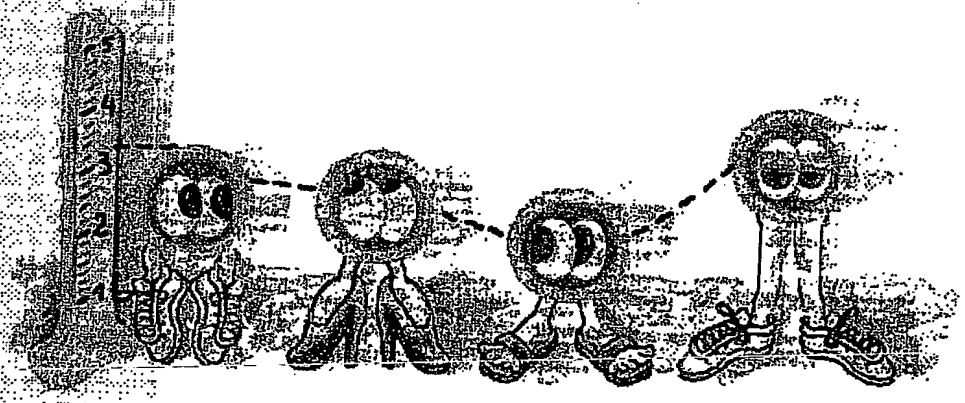


The Embryoguard

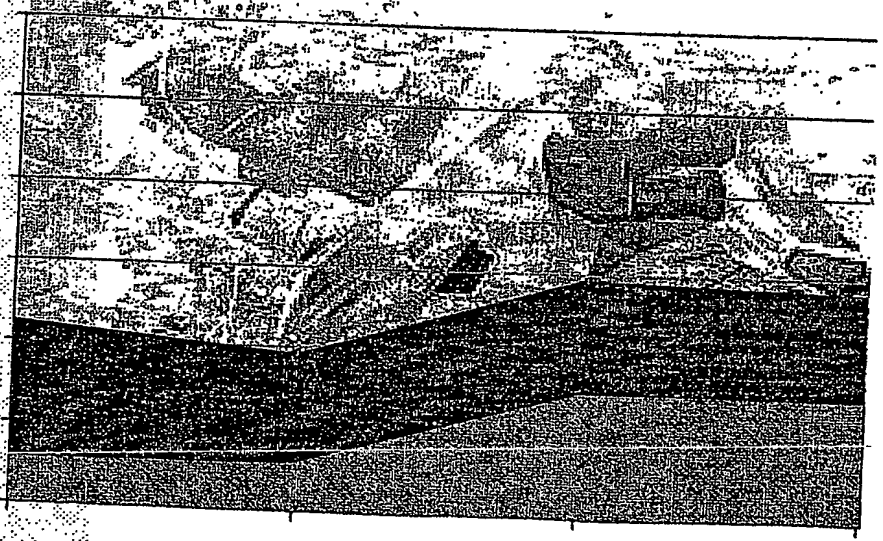
- A new system which contains three microscopic CCD cameras located inside the IVF incubator, including active matching management tool



On- line monitoring & time-laps evaluation of embryos inside incubator

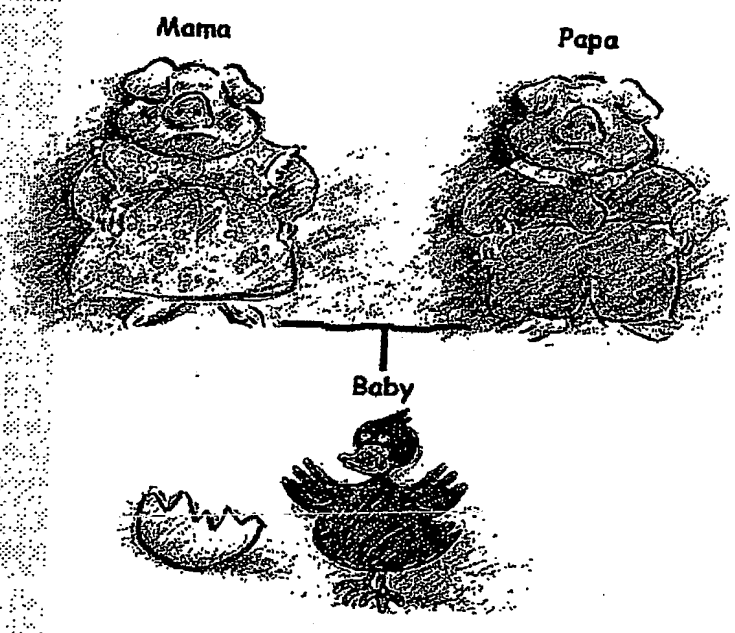


Improved success rates by selecting of embryos based on cleavage timing

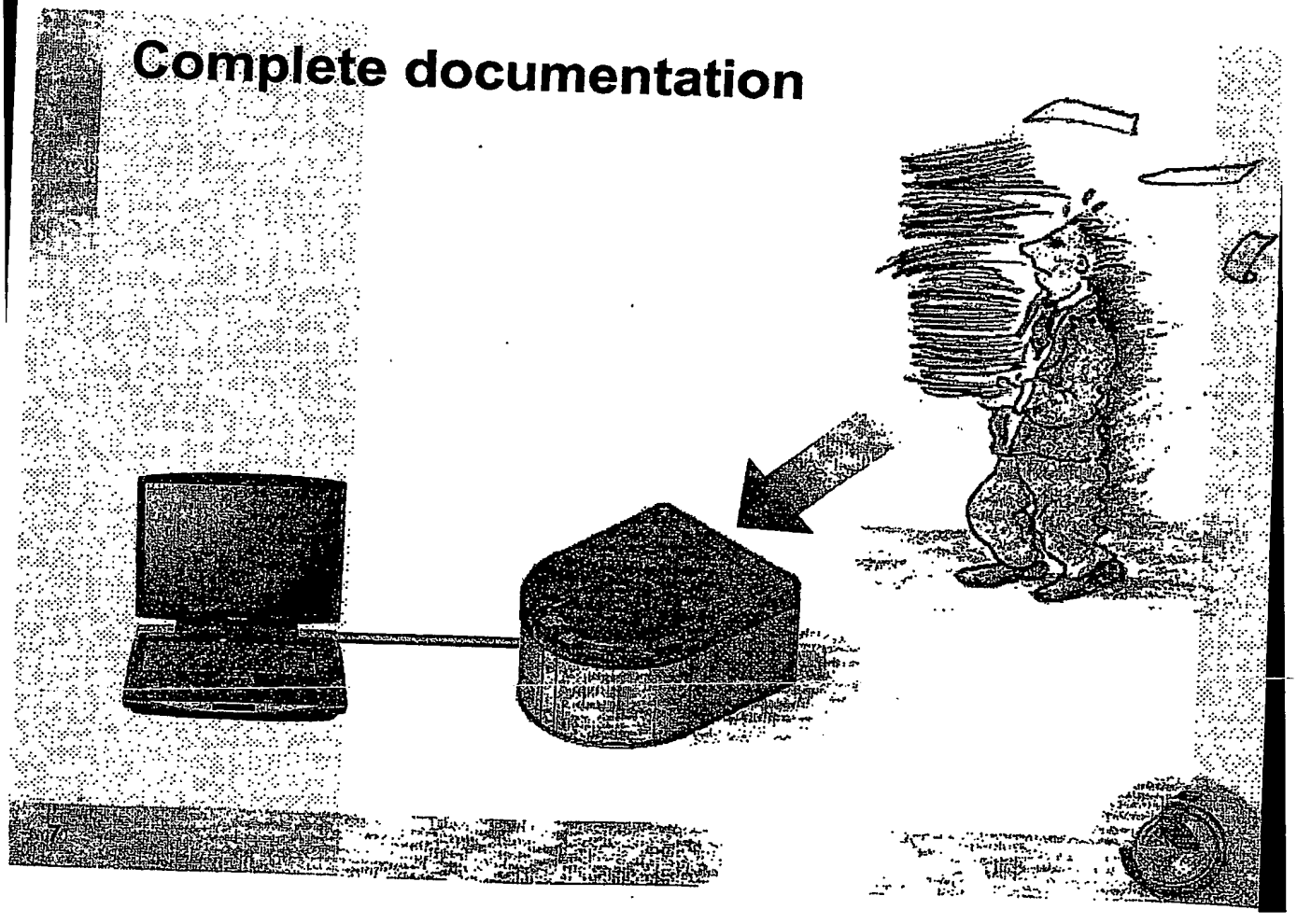


Optimal management of IVF lab procedures

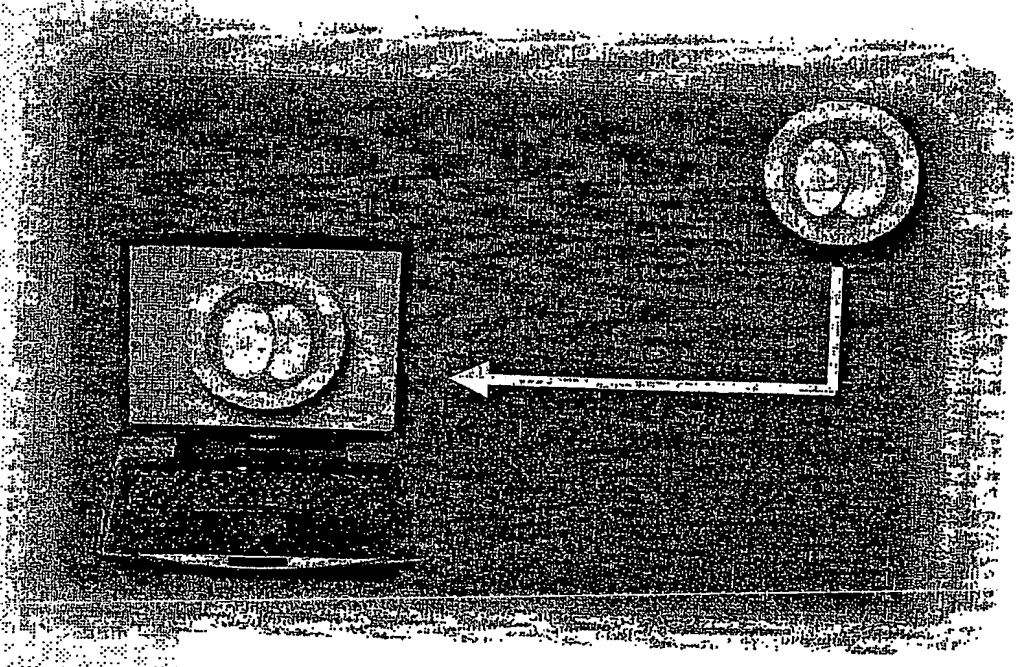
Controlled matching utilizing the Barcode identification system



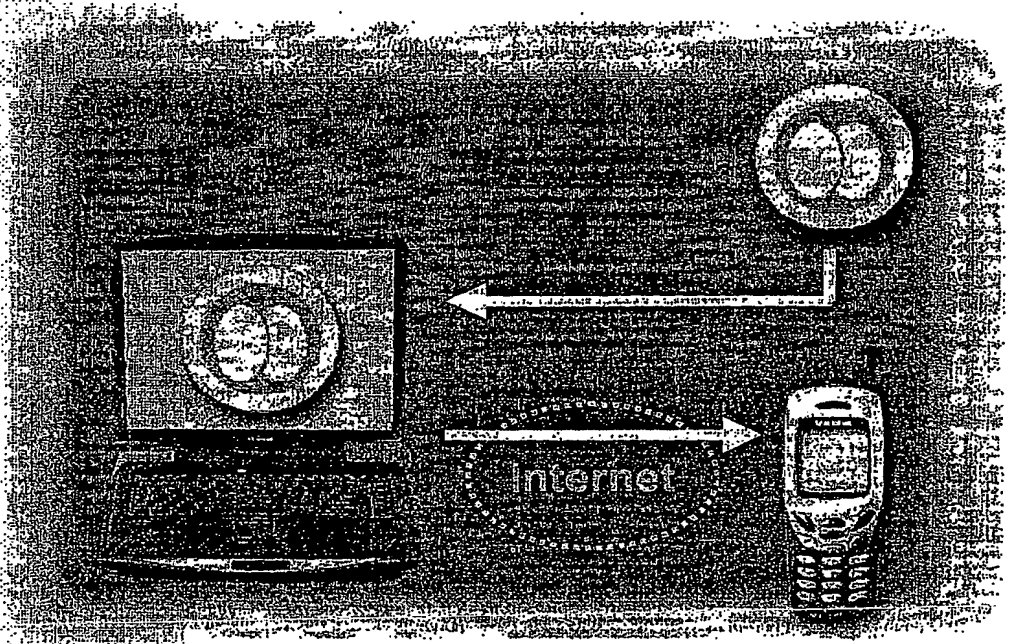
Complete documentation



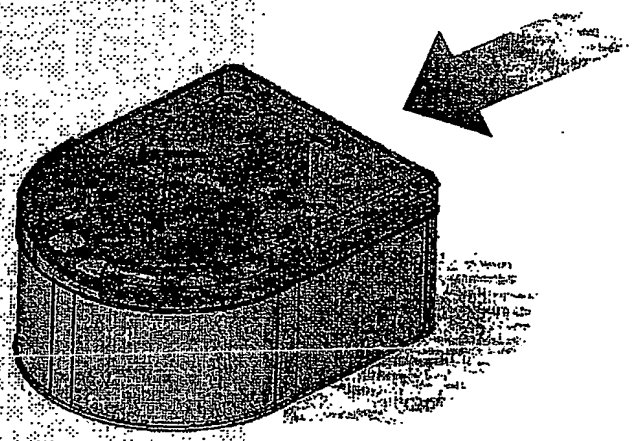
On line control per procedure

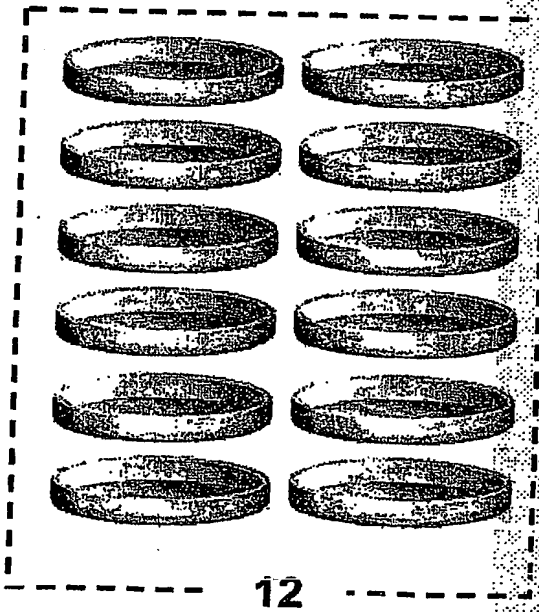


Internet compatibility

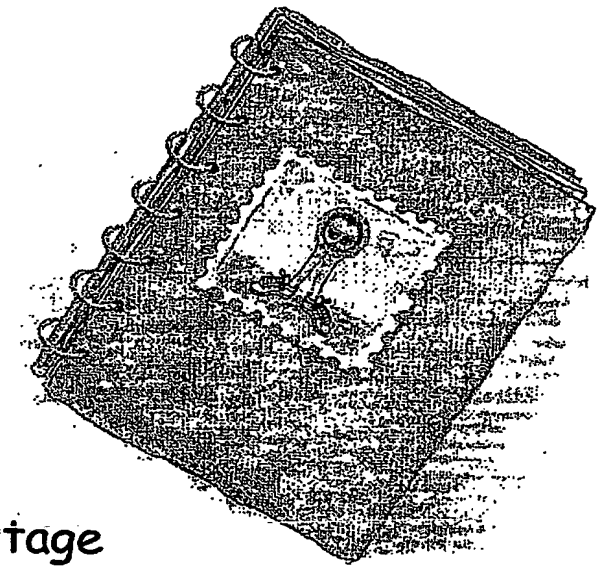


Real time evaluation of up to 12 dishes simultaneously



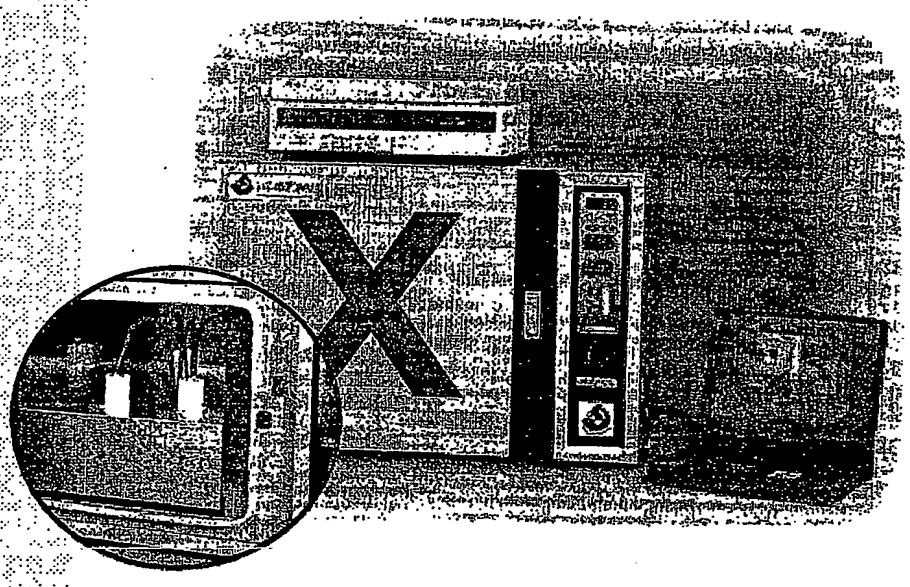


Full robotic 3D movement of CCD microscopic cameras inside the incubator



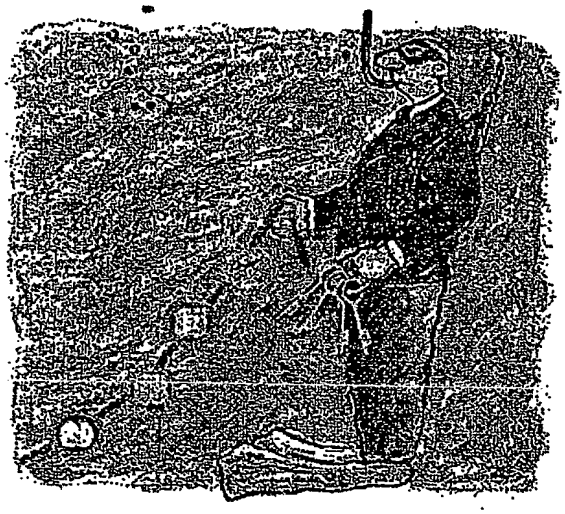
Start your
photo album
from your 2PN stage

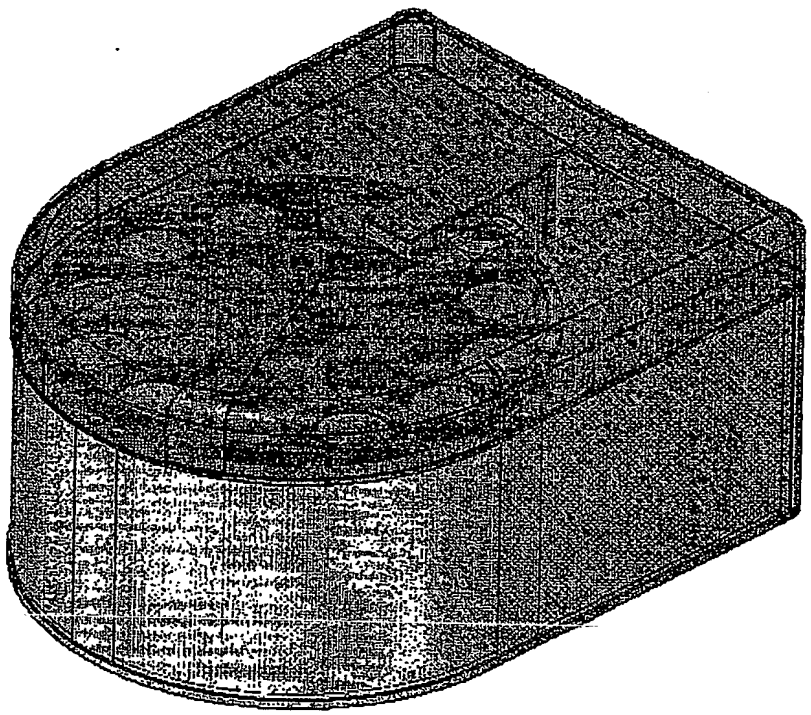
Reduces the need opening the incubator

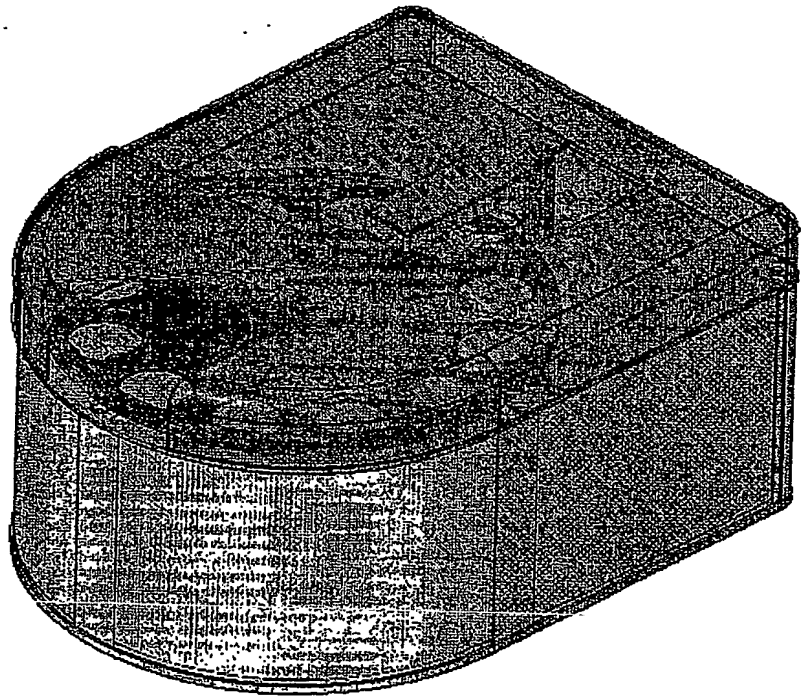


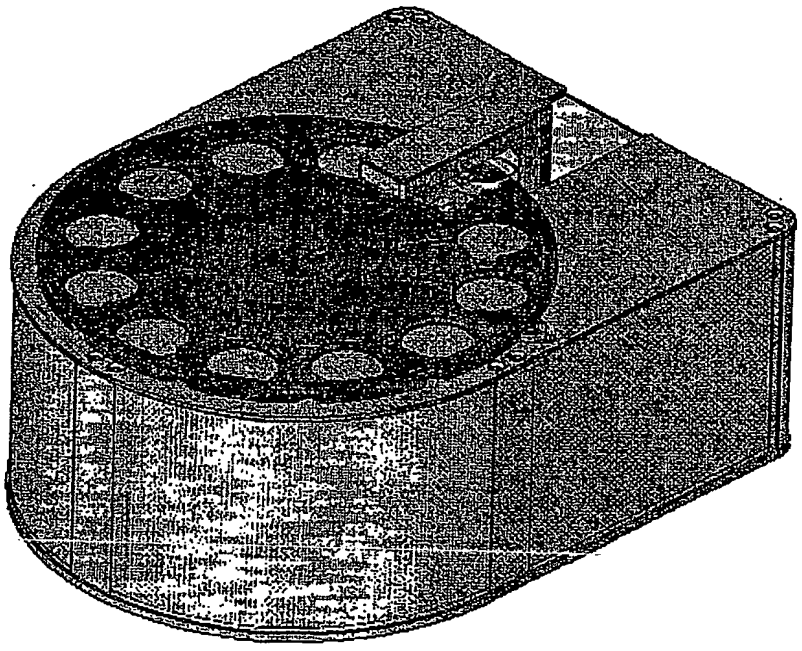
For More Information

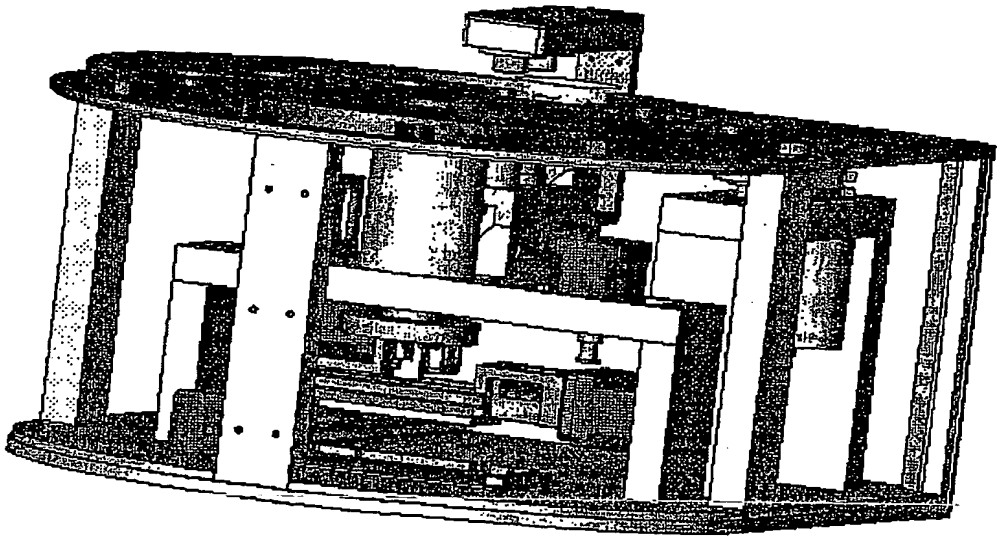
- E-mail: embryoguard@cryo-imt.com
- Site: www.cryo-imt.com

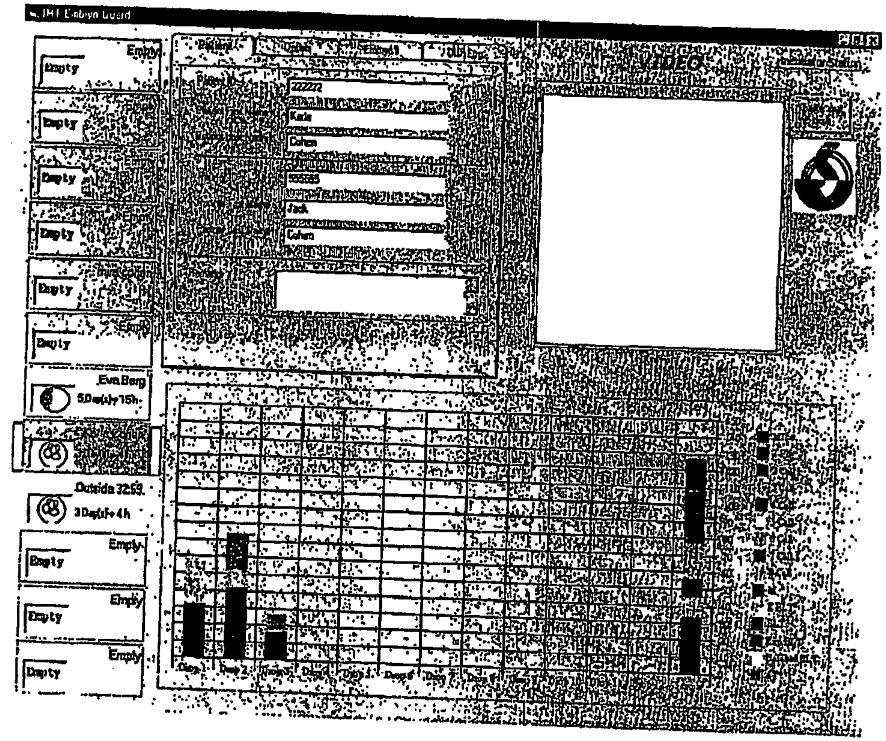








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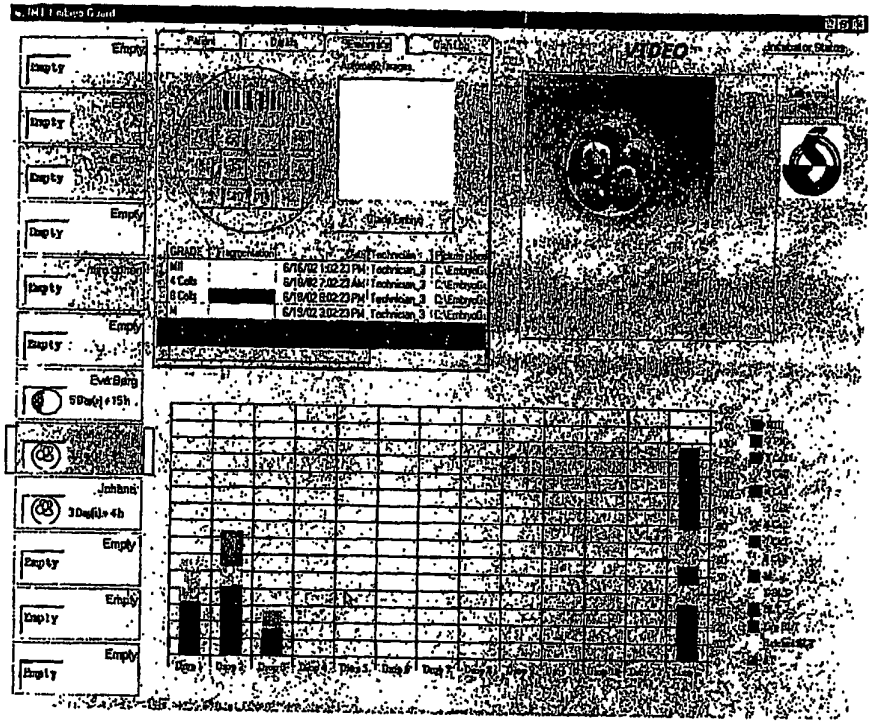
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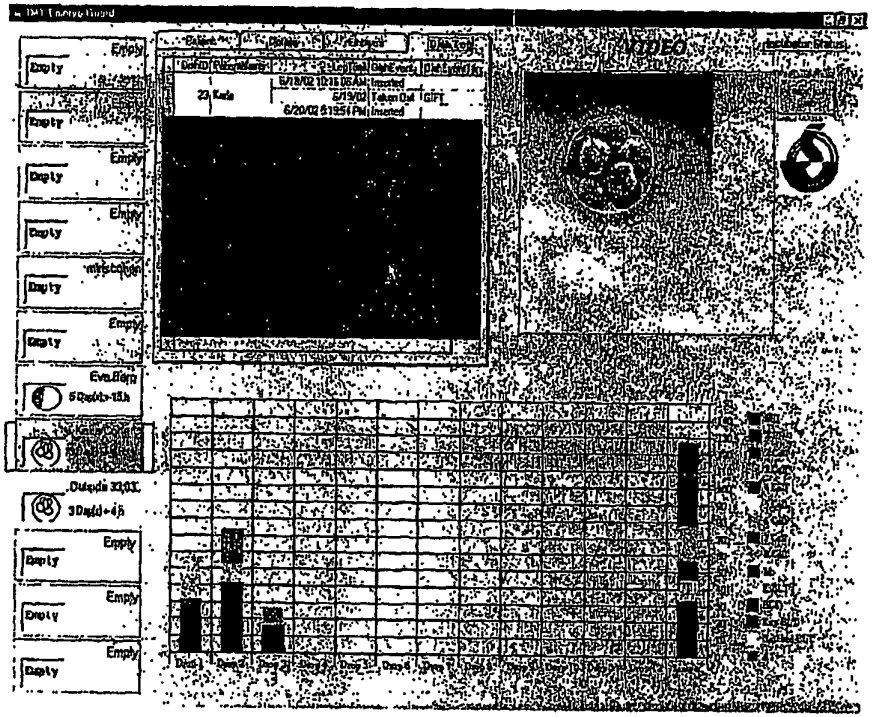
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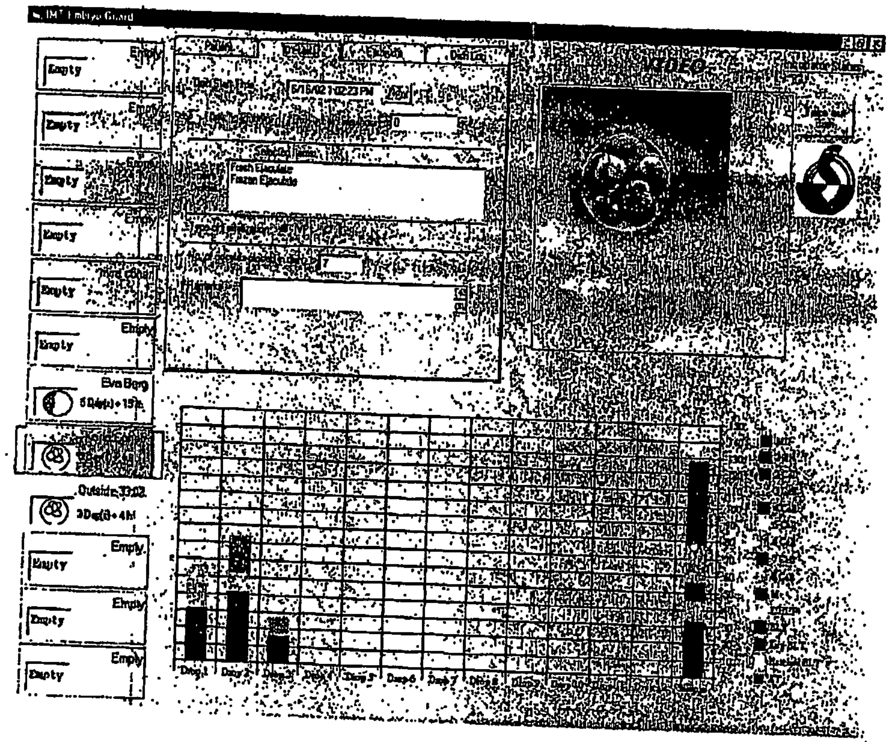
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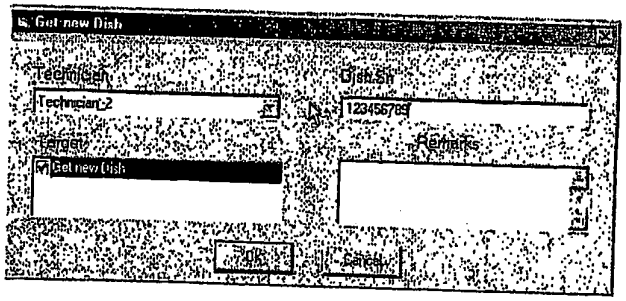
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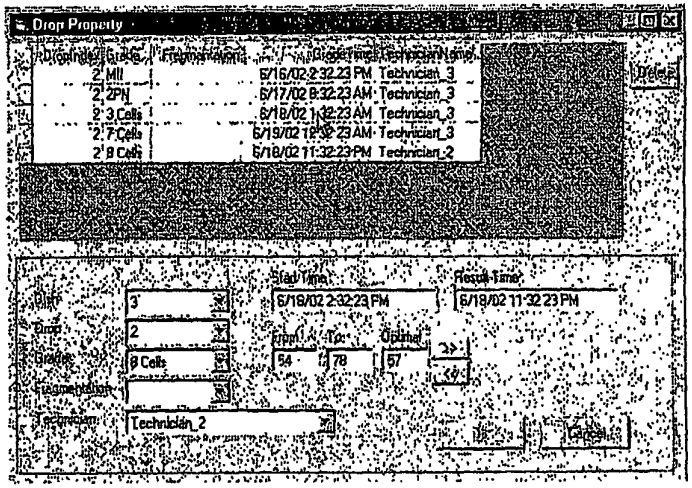
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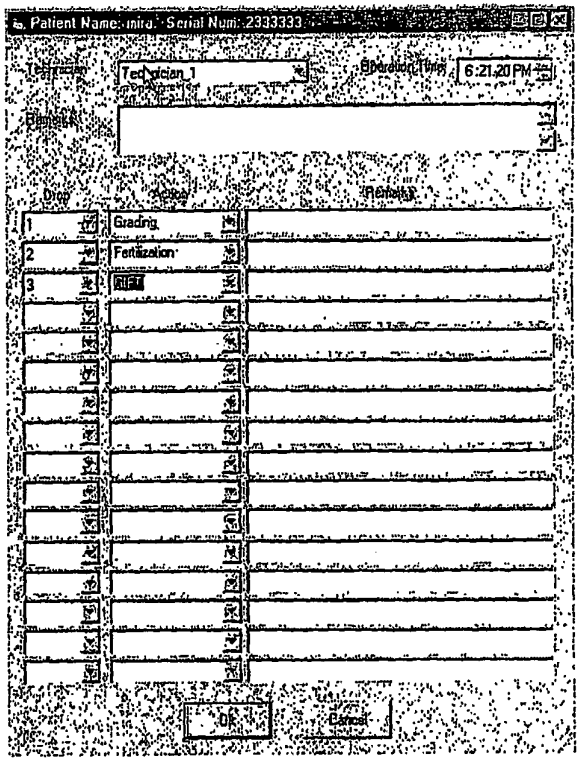
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5.1.1. Bovine Semen

The worldwide use of bovine semen in animal husbandry is enormous, with approximately 100 million doses administered each year. With costs per dose ranging between approximately $4-50/dose, and with a mean of about $10/dose, this total industry has a value of about $ 1 billion per annum. IMT estimates that at a charge of $0.90 per dose, it's market share would be about $100 million per annum.

Use of IMT's MTG™ 525 equipment in the freezing of bull semen has been shown to yield a greater proportion of viable sperm than other conventional methods. This leads to more doses of semen available per ejaculate, a major commercial advantage for the user. In addition, because the IMT technology reduces significantly the damage to sperm cells, it may be possible to achieve successful insemination with smaller doses of semen, an additional advantage of this technology. Field trials are now underway at Cogent Breeding Ltd (UK) to demonstrate this added advantage. Additional potential advantages of IMT technology – the ability to freeze larger volumes of semen in a single container, the ability to refreeze semen specimens after initial thawing, and the freezing of semen specimens that have been sex – sorted - are some additional applications that are being investigated by IMT. All these variations are desirable additional applications of the IMT technology that would have significant operational and commercial benefit to the user. By developing and demonstrating these applications, IMT will be able to secure its place in the market for a long time.

IMT currently has the MTG™ 525 module in routine use in the UK (Cogent). It is also under evaluation in Switzerland. IMT anticipates that an additional 5-6 machines will be placed during the second half of the year 2002.

5.1.2 Equine Semen

Equine semen freezing is not as far advanced in the world market as is bovine semen, but the worldwide market is growing rapidly. Stallion semen is more difficult to freeze than bovine semen, and the semen of about 25% of stallions seem to be resistant to freezing. Success rates for equine artificial insemination is about 40-50%.

As currently practiced, stallions selected for siring are often shipped at sometimes great distances so that natural insemination can take place. This approach is costly and inefficient. In contrast, artificial insemination permits the "banking" of selected stallion semen. Insemination of a mare can take place at the convenience of the owners, even long after the stallion has died. Thus, the use of semen storage offers the horse's owner a form of insurance over his (usually large) investment.

Preliminary studies indicate that IMT freezing technology can improve semen recovery by about 20%. More importantly, semen from stallions that could not be frozen previously has been successfully frozen by this technology and has resulted in pregnancies. This opens up a large and remunerative field for IMT's technology. Based on a $30/dose charge, IMT estimates this current annual market at about $300 million. IMT, in collaboration with Cogent is now continuing to perform field trials in Europe to further document the efficacy of its equipment in the freezing of equine semen. Results are anticipated by the end of year 2002.

5.1.3 Porcine Semen

IMT has recently added the freezing of porcine semen to its growing list of animal artificial insemination applications. Studies in IMT's laboratories indicate that, when using the MTG™ 525 module, there is an excellent, 95% recovery of viable sperm. Field trials in which porcine semen frozen with IMT's technology will be used to inseminate and produce viable offspring are scheduled to commence in the Summer of 2002.

Average production cost of one insemination dose is about $3.5 when produce in the farm (disregarding the boar's genetics). An average farm produces about 15,000 doses annually. This is a market that is in its relatively early stages o development, and its size is difficult to estimate. IMT anticipates that an MTG™ technology will change this field and that there will be newly established artificial insemination farms supplying frozen porcine semen. IMT estimates that it will be able to charge a royalty fee of $0.90 per dose of porcine semen frozen.

[redacted]

Successful pregnancies in cows following double freezing of a large volume of semen

Abstract

The objective of the following paper is to describe a new technology for large volume and double freezing of semen in 12 ml test tube.
Semen from two different bulls was frozen with a new technique using 12 ml test tube and was refrozen after thawing in mini straws. All freezing was done in a "Multi thermal gradient" (MTG) freezing apparatus, which moves the container at a constant velocity (V) through a thermal gradient (G) producing a controlled cooling rate B= (G) x (V).
Each of the two bulls ejaculated were evaluated for post thaw motility in the lab and then in a field trial which was carried out in a split sample mode. We inseminated 105 cows after double freezing/thawing cycle, and another 123 cows were inseminated with semen frozen in mini-straws and a conventional method.
Results showed a 75±5% post thaw motility after freezing a 12ml test tube and 50±5% after second freezing/thawing in mini-straws, respectively.
Controlled vapour freezing showed a 60±10% post thaw motility.
Results of the field trial showed a pregnancy rate of 44% (47/105) for the double freezing group in comparison to 45.5% (56/123) for the controlled group.
These results can be beneficial for large volume freezing, and therefore for bull semen cryobanking in a large volume which will be followed by second freezing in a regular insemination volume.

Bull semen Cryobanking

Cryobanking of semen has had a major impact on dairy cattle genetic breeding
In addition to its role in young bull genetic breeding, cryobanking of bull semen is an important backup for sufficient insemination doses in cases of disease, infertility or mortality.
Freezing and storage of semen is done regularly using mini (¼cc) or midi (½ cc) straws. However, cryobanking of a large number of straws is time consuming, expensive and requires a lot of storage space and liquid nitrogen. An alternative procedure which will reduce these expenses could be the freezing of a whole ejaculate in one test tube (12ml) and only when needed (when the bull is a "proven bull") the test tube will be thawed and then be refrozen in regular mini straws. We describe here the use of a new technology for large volume (whole ejaculate) freezing/thawing and refreezing in mini-straws.

MTG technology

Our novel freezing technology is based on "Multi-thermal gradient (MTG^R,IMT, Israel) (1)" directional solidification and is used mainly for freezing sperm and large

tissue. The semen in the test tube is moved at a constant velocity (V) through a linear temperature gradient (G) so the cooling rate (G x V) and ice front propagation are precisely controlled (Fig. 1).

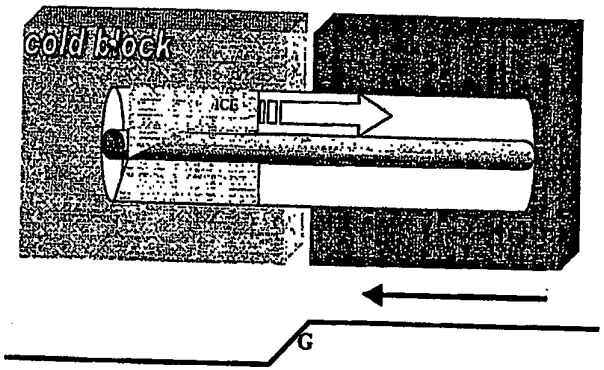

Fig. 1 Schematic design of the MTG freezing

This method also enables the incorporation of controlled seeding into the freezing process. When any liquid is cooled below its freezing point, it remains a liquid, in an unstable super-cooled state, until freezing starts at randomly distributed nucleation sites and spreads throughout the entire volume of the liquid. As discussed above, in the conventional equiaxed method of freezing, ice grows with uncontrolled velocity and morphology, and may disrupt and kill the cells of the samples. Ideally, the velocity of the freezing front should be such that the ice morphology does not disrupt the cells or tissue. However, the rate of cooling appropriate for favorable ice morphology may not be appropriate for other desired outcomes of a sample's freezing protocol. The laterally varying gradient used in our technology allows cooling to proceed at differing rates under varied temperature regimes, thereby facilitating full control over nucleation and ice crystal morphology. This technique allows very precise control of the cooling rate (0.01 to 1000°C/minute) within a large volume.

The freezing apparatus can control ice crystal propagation by changing the thermal gradient (G) or the liquid-ice interface velocity (V) and so optimizing the ice crystal morphology during freezing of cells and tissue. The rate of cooling also affects the

morphology of the intercellular ice crystals (3): morphologies such as closely packed needles kill cells by external mechanical damage (unpublished observation). Thus, maximizing the survival rate of cells subjected to freezing and thawing requires careful control of the freezing process i.e. interface velocity. Using a cryomicroscopy observation we found that survival of sperm shows biphasic curve where at a very slow velocity ice will grow in a planner form which will kill all cells. At higher velocity ice crystals will form secondary branches and survival will increase, however at higher velocity (i.e. 300μm /sec) ice will start to form "needle-like" ice crystals which will decrease PTM, but in a higher velocity will permit very high survival (fig. 2) depending on the space between the ice crystals (4). Finally, at very high velocity (i.e. >3000μm /sec), directional solidification will not occur and survival will decrease.

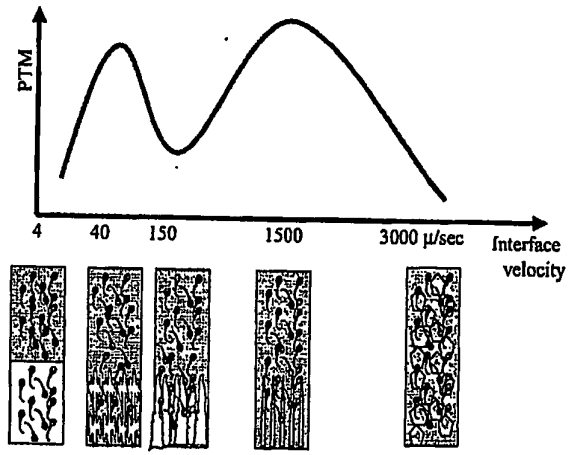

Fig. 2 Effect of interface velocity (V) on ice crystals morphology and sperm post thaw motility (PTM)

Heat transfer problems associated with large volume freezing

In a conventional slow-freezing method, temperature of the chamber is dropped in a controlled stepwise manner. This method is based on using multidirectional (equiaxed) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity and geometrical shape of the container and of the biological material within it (5). The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable. Furthermore, the ambient temperature gradients within the freezing chamber and the unreliability of temperature recording measurements (6) add to the difficulty of achieving the optimal cooling rate in a large volume sample.

Cryobanking of large volume semen

Each of the ejaculate was tested for semen concentration and motility (>70%) before dilution. We used AndroMed® (minitub, Hauptstrabe, Germany) for the semen dilution to have a final concentration of $15x10^6$ sperm/ml.
Freezing of a whole ejaculate was done in a special test tube (12ml) in which the central part is a hollow channel. Heat transfer is opposite to the test tube movement and is parallel to the tube length axis (fig. 1). The empty channel in the middle of the large test tube facilitate directional freezing and rapid thawing in the inner side of the test tube.
Sperm PTM after freezing in a large volume was very high. We found a survival rate of 90-100% (normalized PTM) in the two bulls we cryopreserved in the MTG technique. These results were superior to MTG freezing using mini straws (data not shown), which suggest the benefit of using MTG freezing of large volume for sperm cryopreservation. Results shows a 75±5% post thaw motility after freezing a 12ml test tube and 50±5% after second freezing/thawing in a mini-straws, respectively. Controlled vapour freezing showed a 60±10% post thaw motility which were lower then the results after MTG freezing of mini straws.
The large volume freezing may be very useful for cryobanking of bull semen, for example, AI centre that have a bank of 10,000 straws which are made from 25 ejaculates (400 straws /ejaculate). We calculated that these 10,000 straws will fit into 13 goblets (750 straw/goblet). In comparison, when we freeze a large volume (12ml test tube) the 25 ejaculates will be frozen in 25 test tubes which will be stored only in 2 goblets. This means that we need 6.5 time more goblets using straws in comparision to test tube freezing. In this case, the present method gives a capability to have a bank of "waiting bulls" in some of the AI centers which presently do not use a semen cryobanking. In addition this method will save money in labour and consumables (filling, printing, LN for freezing and for storage etc.).

In conclusion, the MTG technique could be very useful for large volume cryopreservation and double freezing for sperm cryobanking.

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Multigradient

Notes from interview with Amir Arav, 26 June 27-06-2002

Based on US 5,873,254.
Also based on the cryopreservation part of US 60/345,643.

Equine: most stallion sperm can't be frozen. We get higher recovery than others.

Largest volume of semen frozen in prior art in test tube = 5 ml. (Larger volumeof pig semen has been frozen in bags.)

Our innovation: large volume freezing.

Horse prior art: 0.5 ml. One insemination needs four to eight 0.5 ml semen samples.

Why large volume?
For example, for breeding cattle. Genetic breeding. Test young bulls for production. Collect semen. Inseminate heifers. Heifers give birth to heifers. Inseminate the daughters. See how much milk the daughters give. This takes 4 to 5 years. Only one out of every 14 candidate bulls is selected and something may happen to the best bull during the 4-5 years, like the bull may die. Therefore, need to put semen in bank. Store 10,000 to 50,000 0.25 ml straws per bull. It takes 25 days to collect 10,000 straws, and lots of liquid nitrogen for storage.
For volume of one ejaculate: We do one test tube, 12 ml sample, 1:2 dilution instead of 400 to 600 straws, 1:10 dilution. At the end of the 4-5 years, the selected bull's semen is thawed and refrozen in regular straws.

Prior art concept: can't freeze samples bigger than 0.5 ml, can't freeze concentrated sperm.
Prior art: 50,000,000 sperm cells per ml.
Us: 500,000,000 sperm cells per ml.

Equine and boar semen freezing without centrifugation.
Prior art: do centrifugation of semen to remove seminal plasma before freezing.
Sperm concentration in semen is low in stallion and boar: 50,000,000 to 600,000,000 sperm cells per ml.
To remove plasma: centrifuge and wash the sperm. This damages the sperm. Then add extender to get concentration needed for insemination: 1,000,000,000 to 6,000,000,000 sperm cells total.

Us: dilute the plasma. Go down to 20,000,000 sperm cells per ml. Then need 50 ml for one insemination. Large volume freezing allows freezing one insemination (10 ml x 5 or 50 ml x 1) at once.
How?
Multigradient freezing of rotating test tube. Rotate the tube around its longitudinal axis during the freezing.

Advantage: mix the solution in front of the ice front. This dilutes the concentration of salt being expelled from the ice.
Rotating tube also used for warming.
Rotating tube also used for freezing partly filled test tube. Spread solution in annulus to get high surface area.
Rotating the tube gives better thermal contact between the solution and the metal heat exchanger through the walls of the test tube.

The thermal contact with the block is always best on the bottom side of the tube, and that thremal contact gets spread around when the tube is rotated.

If the tube is partly filled with a sample, you wind up with frozen sample on the wall of the tube and air along the axis of the tube. That's better if then you want to lyophilize (freeze dry) the sample.

Rotating the tube keeps the sample mixed and homogeneous during freezing.

Alternative: hollow (double walled) test tube.
Piston sits inside central channel of test tube and removes heat from the central channel. More efficient heat exchange during cooling.
For heating: put hollow test tube in water bath so circulating water flows through the central channel. Warming rate in center of tube and on outside of tube is the same.
Unlike US 5,873,254 seeding, need to inject liquid nitrogen at bottom of test tube. A section at the base of the test tube is arranged to exclude sperm but include liquid, for example by putting glass balls as liquid trap in base of test tube, so only the liquid is frozen for seeding.

Hollow tube can be glass or plastic. Hollow tube need not rotate.
Hollow tube also has a roughened section on side for manual marking.

Warming or thawing small straws (0.25 cc or 0.5 cc samples).
Faster is better, but can't go fast from liquid nitrogen temperatures or heat stress will crack the sample.
OTOH, overheating leads to denaturation, and warm cryoprotectant can damage sperm.
Faster is better to prevent recrystallization at −10°C.
Machine has one block at uniform high temperature: 38°C to 100°C, with 90°C being preferred. Put straw in hole through block. Move straw through the block via the hole at constant velocity. 6mm/sec optimal. Block is 2 cm thick so 3.3333 second duration. Then out to ambient air.
From when straw leaves liquid nitrogen to when straw emerges from block should be less than 50 seconds, preferably about 30 seconds.
Pull straw from liquid nitrogen: goes up to −30°C just by being in ambient air. Put straw through block: in 3 seconds, go to room temperature.

Another way to warm the test tube: like the straws.

Another way to warm a prior art test tube:

Plunge into a water bath warmer than 37°C, with 70°C preferred. So no contact between ice and walls. Then drop contents of test tube into high volume pre-warmed dilution (insemination) solution. Mix.
Alternative: kept he test tube in hot water while "stirring" with the test tube to get uniform thawing.

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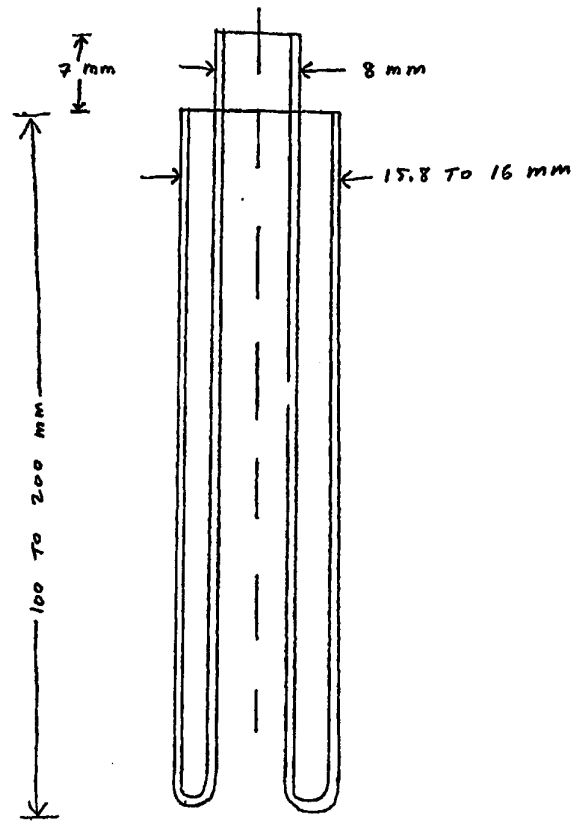
Freezing and thawing test tube with controlled rolling (rotating) system

Inventors: Arav Amir, Meir Uri

We developed a device which has controlled rolling system of round container (i.e. test tube) during freezing and thawing.
The advantageous of this device are:

1. A better heat transfer between the container and the copper blocks.
2. A formation of air bubble in the centre of the container/ a thin layer on the wall of the container.
3. A controlled propagation of ice crystals in parallel to the container wall.
4. A continues mixing of the solution during the freezing and thawing.
5. A preparing of large surface for the purpose of sublimation for freeze drying.

CROSS - SECTION OF HOLLOW TEST TUBE



DOUBLE FREEZING FIRST EXPERIMENT

The first expediment was to find the best dilution rate semen:extender (1:1,1:2, 1:3) for 12 ml. Tubes for dilution 1.1 the semen was diluted with an extender of 10% glycerol for a final concentration of 5% glycerol for dilution 1:2 the semen was diluted with an extender of 7.5% glycerol for a final concentration of 5% glycerol for dilution 1:3 the semen was diluted with an extender of 6.6 % glycerol for a final concentration of 5% glycerol

FREEZING

the semen was taken from two bulls and divided in three parts for the diferents dilutions rates, the semen was frozen in the MTG-516 machine of 12 ml. at a velocity of 1.0 mm/sec. The temperature for block A was 5C and for block B -50C. normal semen was frozen in Digit Cool machine as control.

THAWING

The tubes were held in the air for 140 sec. and then summerged in a water bath at 35C for 25-30 sec. the content of the tube was added to a freezing solution at 37, the semen motility was assessed in the microscop and then cooled down to 5C, the second freezing was doing in 0.5 ml. straws for MTG-510 and 0.25 ml. Straws for Digit Cool machine for control.

RESULTS

First freezing motility

date	bull	1:1 ratio	1:2 ratio	1:3 ratio	nor. semen Control D.C.
08.04.02	Alexis	46.6	63.3	63.3	70.0
	C. kelstein	60.0	70.0	47.5	60.0
AVG		53.3	66.7	55.4	65.0

Second freezing motility

		1:1 ratio	1:2 ratio	1:3 ratio	1:2 ratio Control D.C.
	Alexis	20.0	46.6	25.0	25.0
	C.kelstein	30.0	33.0	30.0	30.0
AVG		25.0	41.8	27.5	27.5

Conclusion: The results show the best dilution rate for freezing in 12 ml. tubes is 1:2 Semen/extender 66.7% and lower results were found for 1:1 and 1:3, 53.3% and 55.4% respectively, the results for the D. Cool machine (control) was 65%. In the second freezing the best result were obtained in 1:2 ratio

DOUBLE FREEZING SECOND EXPERIMENT.

After the first experiment we find that the best results for freezing 12 ml. Tube was 1:2 semen/extender dilution, the second step was to check a big number of bulls based on the results of the first experiment. for better comparison in this experiment we used 0.25 ml. straws within the same sperm concentration 20X10^6 for all treatments.

MOTILITY %

	C. Ryan	L. William	H. Concerto	Alexis	L. Lucan	W. Ginman	C. Dalton	AVG
Raw semen	70	80+	80	80	80+	60	80	78.0
After first freezing tubes	60	65	60	65	60	60	60	61.4
After first freezing D. Cool (control)	65	65	60	70	80	70	80	70.0
After second freezing straws IMT	28	50	15	50	50	40	50	40.0
After second freezing straws D. cool	15	45	40	40	40	60	40	38.6

Conclusions: the average of the results show

1) After first freezing Digit Cool machine is better than first freezing in 12 ml. Tubes in IMT reaching 70% vs 61.4%.
2) After second freezing IMT machine shows a little better results the Digit Cool machine 40.0% vs 38.6%
3) there are individual differences between the bulls in the freezing capacity of the semen and between the two freezing techniks, for example the bull Concerto freeze better in D. cool machine than IMT machine 40% vs 15%
4) we can conclude that is posible to perform a field trial after second freezing with both freezing technics in the second freezing, with those bulls that it semen quality varies from 40% to 50% in D.cool and IMT machine in the second freezing the best bulls for inseminations trials are: L. William, Alexis, L. Lucan, W. Ginman and C Dalton

SORTED SEMEN MOTILITY, DEAD/LIVE RATIO AND MEMEBRANE INTEGRITY AFTER THAWING

The first experiment:
The aim of this experiment was to test the best thawing time for 0.5 ml straws at 90 degrees Celcius.
For straws of 0.25 ml was find in previous work to be 2 seconds in 90 degrees celcius the best thawing time.
At the beginning of each experiment were tested two, three or four straws of 0.5 ml and thawed at differents thawing time. Two test were applied for the assessment of the semen:
1) Visual motility after 5 min. of incubation at 37C
2) Propidium Iodide fluorecent test for dead/live ratio dead cells red colour and live cells blue colour.

The results:

Motility of semen at differents thawing time of 0.5 ml straws at 90C

date	bull	the thawing time in seconds 3	4	5	6
24.06.02	Medowstream			60	30
30.05.02	Shaker		60	65	
31.05.02	Lucky	60	50	60	
01.06.02	Shaker	35	45	60	
02.06.02	Booker	50	40	75	60
05.06.02	Principal	20	30	50	60
07.06.02	Lucky		60	70	60
10.06.02	Courier		60	70	50
17.06.02	Shaker		50	60	40
18.06.02	Courier		30	60	50
AVG		41.3	47.2	65.0	47.1

Propidium Iodide test dead/live sperm ratio

24.06.02	Medowstream			75	67
30.05.02	Shaker		61	65	
31.05.02	Lucky	55	54	55	
01.06.02	Shaker	53	73	73	
02.06.02	Booker	61	79	80	71
05.06.02	Principal	51	68	77	76
07.06.02	Lucky		58	61	60
10.06.02	Courier		68	64	66
17.06.02	Shaker		77	83	82
18.06.02	Courier		63	72	63
AVG		62.6	68.3	70.5	69.3

Results: The average of the results show a better motility and Propidium Iodide test 68% and 70.5% respectively after five sec. thawing in water temperature at 90C for 0.5 ml straws
lower results were find in 3, 4 and 6 sec. thawing.
Conclusion: The best results for both test were five sec. thawing at 90C. for 0.5 ml straws.

[redacted]

SORTED SEMEN MOTILITY, DEAD/LIVE RATIO AND MEMEBRANE INTEGRITY AFTER THAWING

The second experiment:
Based on the first experiment the semen of twelve bulls that normally produce semen for sexing was tested in two temperatures 37C and 90C and two volumes of straws 0.25 ml and 0.5 ml. In order to find the best technic for freezing and thawing semen in the MTG-626 and MTG-550 machines.
The thawing time of 1min at temperature of 37C was the same for 0.25 ml 0.5 ml straws and the thawing time for temperature of 90C was 2 sec for 0.25 ml straws and 5 sec for 0.5 ml straws.
The freezing velocities for 0.5 ml straws was 1.5 mm/sec. With a seeding time of 60 sec.and for the 0.25 ml. straws the freezing velocity was 2.0 mm/sec. with a seeding time of 11 sec.
three test were applied for the assessment of the semen quality.
1) Visual motility after 5 min. incubation
2) Propidium iodide test for dead/live ratio
3) ORT test for membrane integrity, the sperm is incubated in a hipotonic solution for 40 min an then is stained with a fluorecent dye that can identified the affected sperm, we used propidium iodide stain.

The results:

Motility after thawing

date	bull	0.25 ml. 37C.1min	0.25 ml. 90C 2sec	0.50 ml. 37C.1min	0.50 ml. 90C. 5sec
23.05.02	Dazzler	60	60	60	
24.05.02	Meadowstream	50	60	50	65
28.05.02	N. Omar	65	60	50	60
30.05.02	Shaker	60	60	50	60
31.05.02	Lucky	65	60	70	60
01.06.02	Shaker	60	55	60	60
02.06.02	Booker	60	60	60	60
06.06.02	Principal	65	60	60	70
07.06.02	Lucky	55	55	60	70
10.06.02	Courier	60	70	50	70
17.06.02	Shaker	60	70	70	65
18.06.02	Courier	50	60	60	65
AVG		57.5	60.0	57.5	65.3

Propidium Iodide dead/live ratio

		0.25 ml. 37C.1min	0.25 ml. 90C 2sec	0.50 ml. 37C.1min	0.50 ml. 90C. 5sec
23.05.02	Dazzler	59	58	67	76
24.05.02	Meadowstream	62	56	54	74
28.05.02	N. Omar	43	49	76	63
30.05.02	Shaker	54	59	65	58
31.05.02	Lucky	67	60	63	55
01.06.02	Shaker	64	56	76	74
02.06.02	Booker	73	76	67	79
06.06.02	Principal	54	69	55	62
07.06.02	Lucky	64	62	66	70
10.06.02	Courier	79	77	33	64
17.06.02	Shaker	77	76	66	76
18.06.02	Courier	61	69	59	68
AVG		62.3	64.8	60.7	68.3

[redacted]

5

ORT 45 min. Incubation (membrane integrity test)

		0.25 ml. 37C.1min	0.25 ml. 90C. 2sec	0.50 ml. 37C.1min	0.50 ml. 90C. 5sec
23.04.02	Dazzler	43		55	
24.04.02	Meadowstream	31	37	34	43
26.04.02	N. Omar	29	35	45	63
30.04.02	Shaker	40	42	51	57
31.04.02	Lucky	57	44	32	35
01.05.02	Shaker	56	57	44	38
02.05.02	Booker	45	61	35	55
05.05.02	Principal	50	38	35	46
07.05.02	Lucky	35	63	30	43
10.05.02	Courier	55	54	34	51
17.05.02	Shaker	70	53	52	70
18.05.02	Courier	38	44	28	41
AVG		45.8	48.0	39.8	49.3

The results: The best results in motility were for the high thawing temperatures (90C) for the volumes 0.25 ml and 0.5 ml. 65% and 65.3% and the lower results were for the low temperatures (37C) for 0.25 ml and 0.5 ml with 57.5% and 57.8% respectively.

For the Propidium Iodide test (dead/live ratio) the best results were for 0.25 ml and 0.5 ml straws thawed at high temperatures 90C, 64.8% and 65.3% respectively, and lower results were found for 0.25 ml and 0.5 ml straws thawed at lower temperatures 37C, 62.3 and 60.7 respectively

For the ORT test (membrane integrity of the sperm cells) the best results were for 0.25 and 0.5 ml straws thawed at high temperature 90C, 48.0% and 49.3% respectively and lower results were found for 0.25 ml and 0.5 ml thawed at low temperatures 37C, 45.8% and 39.8% respectively,

Conclusions:
The straws thawed at high temperatures shows an improvement in motility, Propidium Iodide, and ORT test it means that less damage is caused to the sperm cells when the thawing is doing at high temperatures 90C (fast thawing), the volume of 0.5 ml straws show a slightly better results in all three test than for 0.25 ml straws when thawed at high temperatures 90C, this results show an advantage for big volumes, that seems to give better protection to the sperm cells under drastic changes in temperature, no differences were found for 0.25 ml and 0.5ml straws when were thawed at low temperatures 37C.except for the ORT test, the better results were for 0.25 ml straws than 0.5 ml straws 45.8% vs 39.8% respectively.

[redacted]

'7th 7:58 (RH) 6

Fine tuning, comparision between 0.5 ml. Straws frozen in MTG-550 vs. 0.25 ml. straw frozen in MTG-625 and Digit Cool machine as controls.

The aim of this experiment was to compare 0.5 ml. Straws frozen in MTG-550 in three differents velocities 1200, 1500, and 1800 mm/sec vs. 0.25 ml. at 2000 mm/sec. the best results for this machine. The temperatures for both machines were 5C for the first block and -50C for the second block, the thawing temperatures of the straws was 37C.

The data is from eleven bulls, that give semen for normal freezing. The concentration of the semen was 20X10^6 per straw, the semen was diluted at room temperature and cooling down at the temperature of 5C, the straws were filled and then frozen in the differents machines.

Results:

The motility of semen, frozen in MTG-550 at differents velocities compared to MTG-625 and Digit Cool machines

Bull	Raw semen motility	V-1200 MTG-550	V-1500 MTG-550	V-1800 MTG-550	V-2000 MTG-625	Digit Cool Machine
Nebulon	80	30	50	5	60	60
Dreamer	80	50	75	5	75	55
C.Laurel	80	40	65	5	60	40
Patton	80	50	75	2	75	60
Luthner	70	50	60	5	60	40
Pierce	80	65	70	55	70	60
Cestrian	70	50	60	5	65	50
C. Cracker	80	30	45	5	50	50
Pezna	40	30	30	2	50	30
C. Herriot	60	50	50	2	75	60
S. Milton	60	50	50	10	40	50
AVG	72.7	45.0	56.4	9.2	60.0	49.5

Conclusions:

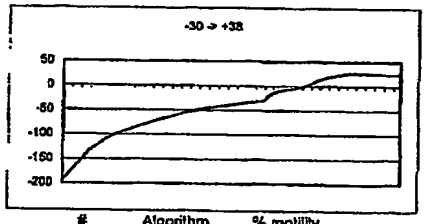
The best results were found for the MTG-625 at a velocity of 2000 mm/sec. 60% and for MTG-550 at a velocity of 1500 mm/sec. with 56.4% motility, lower results were found for Digit Cool machine conventional freezing with 49.5% and for MTG-625 at a velocity of 1200 mm/sec. with 45% motility, the worst results were obtained for MTG-550 at a velocity of 1800 mm/sec. with 9.2% motility.

Bull sperm thawing in thawing machine

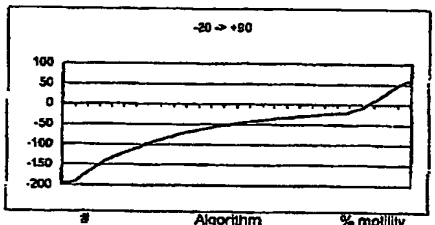
#	T-re	Vel/time	Air time	Microscope						
				% motil	SYBR	PI	SYBR	PI	SYBR	PI
1	90	3 sec.	10	70						
2	90		15	70						
3	90		20	50						
4	90		25	60						
6	90		25	55	89	53	56	77	70	112
5	90		30	70						
7	90		30	70	30	15	28	15	25	14
8	90		30	70	55	40	41	30	25	13
9	90		35	70	35	25	40	25	43	25
10	90		40	45						
11	90		50	15						

Bull semen thawing ...

1. Using water bath



#	Algorithm	% motility
1	-196 > -24(20s) >38	40
2	-196 > -24(20s) >38	65
3	-196 > -24(20s) >38	70
4	-196 > -24(20s) >38	75
5	-196 > -24(40s) >38	50
6	-196 > -24(40s) >38	40
7	-196 > -24(40s) >38	65
8	-196 > -24(40s) >38	70
Average :		62.14286



#	Algorithm	% motility
1	-196 > -24(20s)> +90(2s) >38	70
2	-196 > -24(20s)> +90(2s) >38	77
3	-196 > -24(20s)> +90(2s) >38	75
4	-196 > -24(20s)> +90(2s) >38	75
5	-196 > -24(40s)> +90(2s) >38	75
6	-196 > -24(40s)> +90(2s) >38	76
7	-196 > -24(40s)> +90(2s) >38	75
Average :		74.28571

2. Using new thawing machine

#	Air time	Hot block Temp.	Hot block Time (sec)	% motility
1	10	90	3	70
2	15	90	3	70
3	20	90	3	60
4	25	90	3	60
6	25	90	3	55
5	30	90	3	70
7	30	90	3	70
8	30	90	3	70
9	35	90	3	70

Stallion semen results

11.06.02

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Planer Straw			MTG Tube		
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT
Nemrod	65	60	58.6	49.2	50	38.5	25.3	55	45.8	30.0
Jet set	80	70	59.4	47.1	65	54.0	41.6	65	76.5	50.5
William Curtis	50	50	52.3	35.0	20	25.4	18.1	40	55.0	37.7
Libra K	60	40	46.4	24.6	25	28.3	12.2	60	59.0	34.3
Samhire	60	70	70.0	59.1	60	51.4	33.0	70	68.0	40.3
Mean	67.0	58.0	57.3	43.2	42.0	39.5	26.0	58.0	61.1	38.6

Stallion semen results

10.05.02

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Planer Straw			MTG Tube		
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT
Jet Set	70	40	46.4	24.9	50	49.3	37.1	60	55.1	46.2
Oberon	70	60	48.1	46.2	40	39.2	21.0	50	41.0	26.9
Rubek	80	80	68.2	57.9	60	54.0	24.0	60	51.4	32.1
Nemrod	60	60	64.1	52.1	30	34.3	12.0	50	49.7	21.5
Mean	70.0	60.0	66.7	45.3	45.0	44.2	23.5	55.0	50.1	31.7

Dear Udi

I hope this information is not too late, I have just received your e-mail. Please find attached the results for the latest MTG protocol for stallions. I am applying three tests for post thaw evaluation, namely Osmotic Resistance Testing (a membrane strength stress test), Acridine Orange/Propidium Iodide (a membrane viability test) and motility. None of these tests are unique to us.
The freezing extender I am using contains the following:

Glucose monohydrate 15g
Tri-sodium citrate 0.925g
EDTA 0.925g
Sodium hydrogen carbonate 0.3g
Lactose 55g
Lauryl sulphate 0.375g
Lincospectin 1.0g
Gentamycin 1.25ml
Clarified egg yolk (centrifuged at 10000XG to remove fat) 200ml
Glycerol 30ml (3%)
Water to 1000ml

The clarified egg and low glycerol concentration makes this extender unique to us and could be regarded as specific or the MTG.

The freezing protocol I use is:
Manual seeding
5'C start temp
-50'C end temp
1.0mm/second velocity.

A range of velocities can be applied from 0.6mm/second-3.0mm/second. These should all be protected. Also the start temp can be altered to a range between 25'C-5'C and the end temp can be between -5'C--100'C.

I think you are familiar with all other aspects (tubes etc.). Please do not hesitate to ring me if you need any other assitance. I am here for most of the day.

Regards
Matt

The following section of this message contains a file attachment prepared for transmission using the Internet MIME message format. If you are using Pegasus Mail, or any another MIME-compliant system, you should be able to save it or view it from within your mailer. If you cannot, please ask your system administrator for assistance.

[redacted]

Results

16.06.02-19.06.02

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Planer Straw			MTG Tube			Status
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	
C. R. Gold	25	0	4	0	5	1	0	15	21.5	3.4	fail-fail
Libra-K	80	20	19.4	12.1	20	40.0	33.3	35	43.7	29.4	fail-pass
Samhire	80	60	72.4	46.2	3	31.9	23.3	60	48.6	46.0	fail-pass
Libra-K	80	60	79.1	48.4	20	42.8	33.9	40	57.0	46.0	fail-pass
Mill Law	40	30	42.3	39.2	10	34.5	28.2	30	42.2	35.6	fail-pass
Jestar	80	60	71	59.1	30	29.3	20.1	40	40.3	23.2	fail-pass
Rob Roy	90	80	80.2	79.9	80	75.2	66.3	80	78.1	64.1	fail-pass
Pall Mall	70	20	18.4	0	25	32	16.3	35	38.4	26.2	fail-pass
Jester	60	50	48.6	41.2	30	34.2	22.6	50	53	43.4	fail-pass
Dramiro	70	50	56.2	40	20	24.3	11	40	49.4	41.5	fail-pass
Rubek	60	50	56.8	47.1	35	39.4	21.2	50	44.4	43.9	pass-pass
Rubek	60	60	62.3	51.1	50	49.8	35.9	60	49.2	40.2	pass-pass
Secundus	70	25	39.1	30.2	35	23.5	22	50	53.9	42.6	pass-pass
Schiller	90	60	71	49.8	60.0	43.5	34.7	60.0	64.5	51.2	pass-pass
Ludwig	80	40	39.4	28.4	35	35	28.9	40	36.6	29.4	pass-pass
Schiller	80	60	49.4	37.4	40	37.5	27.6	45	46.3	27.5	pass-pass
Secundus	80	50	62.8	50.1	40	43.2	36.2	50	51.4	37.9	pass-pass
C. R. Gold	0										
Mean	65.8	43.1	48.5	36.7	29.9	34.3	25.0	43.3	45.5	35.1	
Mean (fail-pass)	71.1	47.8	55.3	40.7	26.4	38.2	27.0	45.6	50.1	39.5	
Mean (pass-pass)	74 3	49.3	53 0	42.0	42.1	38.8	29.6	50.7	49.6	39.0	
					Straw	7/17 pass		Tube	18/17 pass		

Stallion semen results

To date 19.05.02

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Straw			MTG Tube		
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT
Samhire					3	31.9	23.3	60	48.6	46.0
Craigmanze Gold	60	0	4	0	5	1	0	15	21.5	3.4
Mill Law					10	34.5	26 2	30	42.2	35.6
Oberon	50	50	54.2		10	7.2	0	10	8.1	0
Seamus	60				10	26 8	12 6	45	41.1	35 1
Dramiro	70	50	58.2	40	20	24 3	11	40	49.4	41 5
Libra K	60				20	40 0	33.3	30	43 7	29.4
Libra K	60				20	42.8	33.9	40	57.0	46.0
William Curtis	60	50	62.3	38.0	20	25 4	18.1	40	53.0	37.7
Eagle	70				25	33	25	60	53.4	28.6
Eagle	70	60	68.4		25	18.1	12 1	60	52.6	49.6
Libra K	70				25	24.1	19	50	49 5	30 3
Libra K	60	40	48 4	24.6	25	28 3	12.2	60	59 0	34 3
Memphis	60				25	28.7	28 7	50	49.1	35.3
Nemrod	55	50	56.1		25	29 6	20.0	40	41.6	29.3
Pall Mall	70	20	18.4	0	25	32	16.3	35	38.4	26.2
Craigmanze Gold	70				30	28.4	27.8	50	44 9	29.6
Jester	80	60	71	59.1	30	29.3	20.1	40	40.3	23.2
Jester	60	50	48 6	41.2	30	34.2	22.6	50	53	43.4
Nemrod	60	60	64.1	52 1	30	34.3	12.0	50	49.7	21.6
Rubek	60	50	48.7		30	29.1	13.4	60	54.8	35.4
Rubek	60	50	53.6		30	34.6	16.4	50	56 9	41.1
Russel	70				30	21.9	17.4	50	57 1	39 4
Ludwig	60	40	39.4	28 4	35	35	28.6	40	56 6	29 4
Rob Roy	80	60	81.1	64	35	47 2	39.7	70	63 6	51 1
Rob Roy	80	60	71 8	62.7	35	41.4	32.2	50	58.2	34.6
Rubekk	60				35	39.4	21.2	50	44 4	43.9
Secundus	70	25	38 1	30.2	35	23.6	22	5	53 9	51.2
Nemrod	70				40	44.9	29.6	60	50	30.7
Oberon	70	60	48 1	45 2	40	39.2	21.0	50	41.0	26.9
Schiller	80	60	49 4	37.4	40	37.5	27.5	45	48.3	27 5
Schiller	90	70	64.2	60	40	49.4	37.6	70	72.3	53 4
Schiller	80	70	69	58.4	40	49.7	35	55	68 4	47 3
Secundus	80	50	62.8	50.1	40	43.2	36.2	50	51.4	37 8
William	60	55	55.0		45	52 3	39.4	60	58 7	39.8
Jet Set	70	40	46.4	24.9	50	49 3	37.1	60	58.1	46.2
Memphis	60	50	47 9		50	53	29.8	60	57.4	37.1
Nemrod	65	60	58 5	49.2	50	38.5	25.3	55	46 6	30.0
Rubekk	60				50	49 8	35.9	60	49.2	40.2
Samhire	80	70	70.0	59.1	50	51.4	33.0	70	68.0	40.3
William Curtis	70				50	49 7	25.0	60	58 4	44.1
Lagos	70				55	43	33.1	65	53.6	34.8
Lagos	80	60	69.1		55	57.6	29.8	50	63.4	50.9
Rubek	80	60	66.2	57.8	60	54.0	24.0	60	51.4	32.1
Schiller	90	60	71	49 8	60	43.5	34.7	60	64.5	51.2
Jet set	80	70	59.4	47 1	65	54.0	41.5	55	76 5	50.5
Rob Roy	90	80	90.2	79 9	80	75.2	56.3	80	78.1	64.1

Stallion semen results

8.06.2002

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Planer Straw			MTG Tube		
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT
Memphis	60	50	47.9		50	53	29.6	60	57.4	37.1
Oberon	50	50	54.2		10	7.2	0	10	8.1	0
Nemrod	55	50	56.1		25	29.5	20.0	40	41.6	29.3
Rubek 1	60	50	45.7		30	29.1	13.4	50	54.8	35.4
Rubek 2	60	50	53.8		30	34.6	16.4	50	56.9	41.1
Eagle	70	60	68.4		25	18.1	12.1	60	52.6	49.8
Lagos	60	60	69.1		55	57.6	29.8	60	63.4	50.0
William	60	55	55.0		45	62.3	39.4	60	58.7	39.8
Mean	59.4	53.1	56.3		33.8	35.2	20.1	48.8	49.2	35.3

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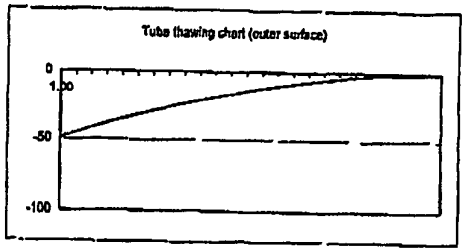

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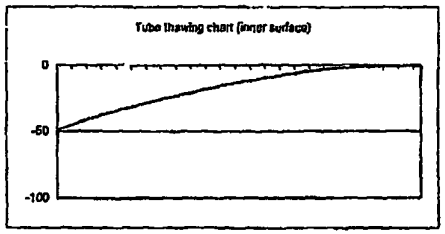

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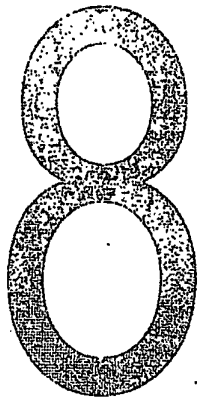

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City: Upper Marlboro State: MD ZIP: 20772

6. Total Number of applications and patents involved: 1

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Amir Arav Meir Uri

(hereinafter called the "assignor(s)"), hereby sell(s), assign(s) and transfer(s) to:

Interface Multigrad Technology Ltd.
3 Hamazmera St.
Ness Ziona 70400
Israel

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EMBRYO GUARD

described and claimed in the following patent applications:

U.S. Provisional Application identified as Attorney docket No. 791/14 and executed the same date as this assignment;

and in and to said Patent Applications, and all original and reissued Patents granted therefor, and all divisions and continuations thereof, including the right to apply and obtain Patents in all other countries, the priority rights under International Conventions, and the Letters Patent which may be granted thereon;

Signed and sealed this 17 day of June 2002

Amir Arav

Meir Uri

Results

16.06.02-19.06 02

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Planer Straw			MTG Tube			Status
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	
C. R. Gold	25	0	4	0	5	1	0	15	21.6	3.4	fail-fail
Libra-K	80	20	19.4	12.1	20	40.0	33.3	35	43.7	29.4	fail-pass
Samhire	60	60	72.4	46.2	3	31.9	23.3	60	48.6	46.0	fail-pass
Libra-K	80	60	79.1	48.4	20	42.8	33.9	40	57.0	46.0	fail-pass
Mill Law	40	30	42.3	39.2	10	34.5	26.2	30	42.2	35.6	fail-pass
Jester	90	60	71	59.1	30	29.3	20.1	40	40.3	23.2	fail-pass
Rob Roy	90	80	90.2	79.9	80	75.2	66.3	80	78.1	64.1	fail-pass
Pall Mall	70	20	18.4	0	25	32	16.3	35	38.4	26.2	fail-pass
Jester	60	60	48.6	41.2	30	34.2	22.6	60	53	43.4	fail-pass
Dramiro	70	50	56.2	40	20	24.3	11	40	49.4	41.5	fail-pass
Rubek	60	50	58.8	47.1	35	39.4	21.2	50	44.4	43.9	pass-pass
Rubek	60	60	52.3	51.1	50	49.8	35.9	60	49.2	40.2	pass-pass
Secundus	70	25	39.1	30.2	35	23.5	22	50	53.9	42.6	pass-pass
Schiller	80	60	71	49.8	60.0	43.5	34.7	60.0	64.5	51.2	pass-pass
Ludwig	80	40	39.4	28.4	35	35	28.9	40	38.6	29.4	pass-pass
Schiller	80	60	49.4	37.4	40	37.5	27.5	45	46.3	27.5	pass-pass
Secundus	80	50	62.8	50.1	40	43.2	36.2	50	51.4	37.9	pass-pass
C. R. Gold	0										
Mean	65.8	43.1	48.5	36.7	29.9	34.3	25.0	43.3	45.5	35.1	
Mean (fail-pass)	71.1	47.8	55.3	40.7	26.4	38.2	27.0	45.6	50.1	39.5	
Mean (pass-pass)	74.3	49 3	53 0	42.0	42.1	38.8	29.5	50.7	49.5	39.0	
					Straw	7/17 pass		Tube	18/17 pass		

Stallion semen results

To date 19.05.02

Stallion	Pre-freeze motility	Chilled semen after 30hrs			Pisner Straw			MTG Tube		
		%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT	%motility	AO/PI %live	ORT
Samhire					3	31.9	23.3	50	48.6	46.0
Craigmanco Gold	60	0	4	0	5	1	0	15	21.5	3.4
Mill Law					10	34.5	26.2	30	42.2	35.6
Oberon	50	50	54.2		10	7.2	0	10	8.1	0
Seamus	60				10	26.6	12.6	45	41.1	33.1
Dramiro	70	50	65.2	40	20	24.3	11	40	49.4	41.5
Libra K	60				20	40.0	33.3	30	43.7	29.4
Libra K	60				20	42.8	32.9	40	57.0	46.0
William Curtis	50	50	52.3	36.0	20	35.4	18.1	40	55.0	37.7
Eagle	70				25	33	25	60	63.4	28.8
Eagle	70	60	68.4		25	18.1	12.1	60	52.8	49.6
Libre K	70				25	24.1	19	50	49.8	30.3
Libra K.	60	40	45.4	24.6	25	28.3	12.2	60	58.0	34.3
Memphis	60				25	28.7	29.7	50	49.1	35.3
Nemrod	55	50	56.1		25	29.5	20.0	40	41.6	29.3
Pall Mall	70	20	18.4	0	25	32	16.3	35	38.4	26.2
Craigmanco Gold	70				30	28.4	27.8	50	44.9	29.8
Jester	90	60	71	59.1	30	29.3	20.1	40	40.3	23.2
Jester	60	60	48.6	41.2	30	34.2	22.6	60	63	43.4
Nemrod	60	60	64.1	52.1	30	34.3	12.0	50	49.7	21.6
Rubek	60	50	45.7		30	29.1	13.4	50	54.6	35.4
Rubek	60	50	53.8		30	34.8	16.4	50	55.9	41.1
Russel	70				30	21.9	17.4	50	57.1	39.4
Ludwig	60	40	39.4	29.4	35	35	28.9	40	38.6	29.4
Rob Roy	90	60	81.1	64	35	47.2	39.7	70	63.6	51.1
Rob Roy	90	60	71.6	62.7	35	41.4	32.2	55	56.2	34.8
Rubekk	60				35	38.4	21.2	50	44.4	43.9
Secundus	70	25	39.1	30.2	35	23.5	22	5	53.9	51.2
Nemrod	70				40	44.9	29.6	50	50	30.7
Oberon	70	60	48.1	45.2	40	39.2	21.0	50	41.0	26.9
Schiller	80	60	49.4	37.4	40	37.5	27.5	45	46.3	27.5
Schiller	90	70	84.2	60	40	49.4	37.5	70	72.3	53.4
Schiller	80	70	69	58.4	40	49.7	36	60	65.4	47.3
Secundus	80	60	62.8	60.1	40	43.2	36.2	50	51.4	37.8
William	60	55	65.0		45	52.3	39.4	60	58.7	39.8
Jet Set	70	40	48.4	24.9	50	49.3	37.1	60	58.1	45.2
Memphis	60	50	47.9		50	53	29.5	60	57.4	37.1
Nemrod	65	60	58.6	49.2	50	38.5	25.3	55	48.8	30.0
Rubekk	60				50	49.8	35.9	50	49.2	40.2
Samhire	80	70	70.0	59.1	50	51.4	33.0	70	68.0	40.3
William Curtis	70				50	49.7	25.0	60	56.4	44.1
Lagos	70				55	43	30.1	65	53.6	34.6
Lagos	60	60	69.1		55	57.6	29.8	60	63.4	50.0
Rubek	80	60	68.2	57.9	60	54.0	24.0	60	51.4	32.1
Schiller	90	60	71	49.6	60	43.6	34.7	60	64.5	51.2
Jet set	80	70	69.4	47.1	65	54.0	41.6	65	76.8	59.6
Rob Roy	90	80	90.2	79.9	60	75.2	58.3	80	78.1	64.1